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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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CONTENTS

No. 1. August, 1931

PAGE
Thirty-third Report of the Marine Biological Laboratory 1
TYLER, ALBERT
The Relation between Cleavage and Total Activation in Artificially Activated Eggs of Urechis 45
SMITH, GEORGE MILTON
The Occurrence of Melanophores in certain Experimental Wounds of the Goldfish (Carassius auratus) 73
DICKMAN, ALBERT
Studies on the Intestinal Flora of Termites with reference to their Ability to Digest Cellulose 85
LUTZ, BRENTON R.
The Innervation of the Stomach and Rectum and the Action of Adrenaline in Elasmobranch Fishes 93
JOHNSON, GEORGE E., AND NELSON J. WADE
Laboratory Reproduction Studies on the Ground Squirrel, Citellus tridecemlineatus pallidus, Allen 101
BURKENROAD, M. D.
A New Pentamerous Hydromedusa from the Tortugas 115
ONORATO, A. R., AND H. W. STUNKARD
The Effect of certain Environmental Factors on the Development and Hatching of the Eggs of Blood Flukes 120

No. 2. October, 1931

PATTERSON, J. T.
Continuous versus Interrupted Irradiation and the Rate of Mutation in Drosophila 133
TORVIK, M. M.
Genetic Evidence for Diploidism of Biparental Males in Habrobracon 139
WERNER, ORILLA STOTLER
The Chromosomes of the Domestic Turkey 157
ALEXANDER, GORDON
The Significance of Hydrogen Ion Concentration in the Biology of Euglena gracilis Klebs 165

38883

Redfield, A. C., and M. Florkin
The Respiratory Function of the Blood of Urechis caupo 185
Scott, W. J.
Oxygen and Carbon Dioxide Transport by the Blood of the Urodele, Amphiuma tridactyla 211
Mast, S. O.
Movement and Response in Difflugia with special reference to the Nature of Cytoplasmic Contraction 223
Stunkard, H. W.
The Effect of Dilution of Sea Water on the Activity and Longevity of Certain Marine Cercariae 242

No. 3. December, 1931

Harvey, E. Newton
The Tension at the Surface of Marine Eggs, especially those of the Sea Urchin, Arbacia 273
Taylor, G. Wellford, and E. Newton Harvey
The Theory of Mitogenetic Radiation 280
Whitaker, D. M.
Some Observations on the Eggs of Fucus and upon their Mutual Influence in the Determination of the Developmental Axis 294
Coe, Wesley R.
Spermatogenesis in the California Oyster (Ostrea lurida) 309
Blum, H. F., and G. C. McBride
Studies of Photodynamic Action, III. The difference in mechanism between photodynamic hemolysis and hemolysis by non-irradiated eosine 316
Carothers, E. Eleanor
The Maturation Divisions and Segregation of Heteromorphic Homologous Chromosomes in Acrididae (Orthoptera) 324
Adolph, Edward F.
The Size of the Body and the Size of the Environment in the Growth of Tadpoles 350
Adolph, Edward F.
Body Size as a Factor in the Metamorphosis of Tadpoles 376
Jahn, Theo. L.
Studies on the Physiology of the Euglenoid Flagellates, III. The effect of hydrogen ion concentration on the growth of Euglena gracilis Klebs 387
Hall, Victor E.
The Muscular Activity and Oxygen Consumption of Urechis caupo 400

BAUMBERGER, J. P., AND L. MICHAELIS
The Blood Pigments of Urechis caupo 417
FLORKIN, MARCEL, AND ALFRED C. REDFIELD
On the Respiratory Function of the Blood of the Sea Lion . . . 422
ROOT, R. W.
The Respiratory Function of the Blood of Marine Fishes 427
HALL, F. G.
The Respiration of Puffer Fish 457
TANG, PEI-SUNG
The Rate of Oxygen Consumption of Asterias Eggs before and after Fertilization 468
FAULKNER, G. H.
Notes on the Feeding Mechanism and on Intestinal Respiration in Chaetopterus variopedatus 472
WHITING, P. W.
Diploid Male Parts in Gynandromorphs of Habrobracon 478
WHITING, P. W., AND M. F. STANCATI
A Gynandromorph of Habrobracon from a Post-reduced Binucleate Egg 481
WILLIAMS, MARY MORRISON, AND M. H. JACOBS
On Certain Physiological Differences between Different Preparations of So-Called "Chemically Pure" Sodium Chloride . . . 485
WELSH, JOHN H.
Specific Influence of the Host on the Light Responses of Parasitic Water Mites 497
PARPART, ARTHUR K.
Is Osmotic Hemolysis an All-or-None Phenomenon? 500
PARPART, A. K., W. R. AMBERSON AND D. R. STEWART
The Determination of Hemoglobin Concentration in Dilute Solutions 518

Vol. LXI, No. 1 August, 1931

THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

Thirty-Third Report for the Year 1930—
Forty-Third Year

I. Trustees and Executive Committee (as of August 12, 1930) 1
Library Committee 3
II. Act of Incorporation 3
III. By-Laws of the Corporation 3
IV. Report of the Treasurer 5
V. Report of the Librarian 9
VI. Report of the Director 10
Statement 10

Addenda:

1. The Staff, 1930 15
2. Investigators and Students, 1930 17
3. Tabular View of Attendance 28
4. Subscribing and Coöperating Institutions, 1930 28
5. Evening Lectures, 1930 29
6. Shorter Scientific Papers, 1930 31
7. Members of the Corporation 34

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MARINE BIOLOGICAL LABORATORY
WOODS HOLE, MASS.
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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years, and in addition there shall be two groups of Trustees as follows: (*a*) Trustees *ex officio,* who shall be the President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (*b*) Trustees Emeritus, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeritus shall each have the same right to vote as the regular Trustees.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen: Herewith is submitted my report as Treasurer of the Marine Biological Laboratory for the year 1930.

The accounts have been audited by Seamans, Stetson and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and is open to inspection by members of the Corporation.

At the end of the year 1930, the book value of the General Endowment Fund in the hands of the Central Hanover Bank and Trust Company (of New York) as Trustee was $908,915 in securities and $34.50 in cash. The actual market value of the securities in this fund on the 9th day of May taking the mortgages at face value, was $931,981.25, showing a very satisfactory appreciation of the value over cost.

The book value of the Library Fund was $199,922.50 in securities and $77.50 in cash. The actual market value of the securities on May 9th was $203,031.25.

At the end of the year the Lucretia Crocker Fund consisted of securities of the book value of $4,707.59 and $374.32 in cash.

The Bio Club Scholarship Fund consisted of a mortgage participation of $2,000 and cash of $31.28, the Reynold A. Spaeth Memorial Lecture Fund of $3,000 in mortgage securities and cash of $75.46.

The Reserve Fund, consisting of the proceeds of the sale of part of the Bar Neck property to the Woods Hole Oceanographic Institution, consisted at the end of the year of bonds of the book value of $20,868.75 and cash of $3,090.55, of which cash $3,000 was later paid out under the contract, leaving net proceeds of the transaction of $20,959.30 which is being held with its income to meet maturing mortgage obligations or for such other purposes as the Trustees may decide.

The Retirement Fund at the end of the year consisted of $15,800 invested in mortgage participations, less an overdraft of $9.73, leaving $15,790.27.

The land, buildings, equipment and library, excluding the Devil's Lane and Gansett property, represented an investment of $1,617,086.71, less depreciation of $246,625.64, or a net amount of $1,370,461.07.

Current expenses including depreciation exceeded income for the year by $3,767.25.

Over $29,000 was expended from current funds on buildings, equipment and library.

At the end of the year the Laboratory owed $1,640.99 on accounts payable and $27,000 on bonds secured by mortgage.

Following is the Balance Sheet as of December 31, 1930, and the condensed statement of income and outgo for the year, also the surplus account.

EXHIBIT A

MARINE BIOLOGICAL LABORATORY BALANCE SHEET,

DECEMBER 31, 1930

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank & Trust Company (of New York) Trustee—Schedules I-a and I-b		$1,108,949.50	
Securities and Cash—Minor Funds—Schedule II		10,188.65	$1,119,138.15
Plant Assets:			
Land—Schedule IV	$ 97,103.05		
Buildings—Schedule IV	1,207,354.03		
Equipment—Schedule IV	155,222.28		
Library—Schedule IV	157,407.35	$1,617,086.71	
Less Reserve for Depreciation		246,625.64	
		$1,370,461.07	
Securities and Cash in Reserve Fund		23,675.43	
Cash in Dormitory Building Fund		818.96	$1,394,955.46
Current Assets:			
Cash		$ 18,010.39	
Accounts—Receivable		18,902.69	
Inventories:			
Supply Department	$ 29,063.54		
Biological Bulletin	7,951.85	37,015.39	
Investments:			
Devil's Lane Property	$ 37,780.91		
Gansett Property	2,273.34		
Stock in General Biological Supply House, Inc.	12,700.00		
Retirement Fund Assets	15,790.27	68,544.52	
Prepaid Insurance		3,992.51	$146,465.50

Liabilities

Endowment Funds:		
General Endowment Funds—Schedule III	$1,108,949.50	
Minor Endowment Funds—Schedule III	10,188.65	$1,119,138.15
Plant Funds:		
Donations and Gifts—Schedule III	$1,025,548.61	
Other Investments in Plant from Gifts and Current Funds	364,406.85	
	$1,389,955.46	
Mortgage, Danchakoff Estate	2,000.00	
Accrued Charges on Sale of Bar Neck Land	3,000.00	$1,394,955.46

Current Liabilities and Surplus:			
Mortgage, Devil's Lane Property		$ 25,000.00	
Accounts—Payable		1,640.99	
Woods Hole Oceanographic Institution:			
Amount received for Purchase of Books for their Library	$2,500.00		
Less Expenditures	2,147.07	352.93	
Items in Suspense (Net)		70.49	
		$ 27,064.41	
Current Surplus—Exhibit C		119,401.09	$146,465.50

EXHIBIT B

Marine Biological Laboratory Income and Expense,

Year Ended December 31, 1930

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund		$ 48,020.46		$ 48,020.46
Library Fund		9,270.24		9,270.24
Gifts		500.00		500.00
Instruction	8,110.03	10,230.00		2,119.97
Research	4,069.37	16,261.06		12,191.69
Evening Lectures	135.48		135.48	
Biological Bulletin and Membership Dues	7,557.17	9,421.18		1,864.01
Supply Department—Schedule V	62,030.00	62,162.82		132.82
Mess—Schedule VI	30,943.36	32,973.28		2,029.92
Dormitories—Schedule VII	31,188.42	13,764.28	17,424.14	
(Interest and Depreciation charged to above three Departments. See Schedules V, VI, and VII)	*35,424.79*			35,424.79
Dividends, General Biological Supply House, Inc.		2,540.00		2,540.00
Rent, Danchakoff Cottages	634.11	1,039.00		404.89
Rent, Microscopes		462.50		462.50
Rent, Garage, Railway, etc.		154.90		154.90
Rent, Newman Cottage	137.27	150.00		12.73
Rent, Janitor's House	35.84	422.50		386.66
Sale of Duplicate Library Sets		2,198.13		2,198.13
Interest on Bank Balances		529.87		529.87
Sundry Items		10.64		10.64
Maintenance of Plant:				
New Laboratory Expense	16,839.26		16,839.26	
Chemical and Special Apparatus	10,783.01		10,783.01	
Maintenance, Buildings and Grounds	9,892.82		9,892.82	

Library Department Expenses	8,912.66		8,912.66	
Carpenter Department Expenses	1,516.91		1,516.91	
Truck Expenses	851.48		851.48	
Sundry Expenses	772.69		772.69	
Bar Neck Property Expenses	162.54		162.54	
Workmen's Compensation Insurance	592.59		592.59	
General Expenses:				
Administration Expenses	14,509.68		14,509.68	
Endowment Fund Trustee	787.50		787.50	
Interest on Loans	120.00		120.00	
Bad Debts	317.98		317.98	
Naples Zoological Station, for Research	250.00		250.00	
Mosquito Fund Contribution	100.00		100.00	
Reserve for Depreciation	38,052.73		38,052.73	
Excess of Expenses over Income carried to Current Surplus—Exhibit C		3,767.25		3 767.25
	$213,878.11	$213,878.11	$122,021.47	$122,021.47

EXHIBIT C

MARINE BIOLOGICAL LABORATORY, CURRENT SURPLUS ACCOUNT

YEAR ENDED DECEMBER 31, 1930

Balance, January 1, 1930			$119,933.29
Add:			
Reserve for Depreciation charged to Plant Funds			38,052.73
Income from Retirement Fund			603.30
Cash received from Sale of Plant Assets deposited in Current Cash (Motor-Boat)			50.00
			$158,839.32
Deduct:			
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV,			
Buildings	$ 479.17		
Equipment	5,464.23		
Library Books, etc.	23,099.38		
		$29,042.78	
Purchase of Books from Balance of General Education Board Gift of $50,000.00 for Purchase of Books		5,708.20	
Payment of Pensions from Retirement Fund		720.00	
Excess of Expenses over Income for Year as shown in Exhibit B		3,767.25	39,238.23
Balance, December 31, 1930—Exhibit A			$119,401.09

Respectfully submitted,

LAWRASON RIGGS, JR.,

Treasurer.

V. THE REPORT OF THE LIBRARIAN

The important feature of 1930, which was the establishment of a regular endowment fund for the Library which would ordinarily give, along with the usual laboratory allowance, about $24,000 annually, was included in the report of last year. A general statement of the future apportionment of this sum as there given has been carried out in fact. A very important addition occurred in the spring, however, when the Director of the Woods Hole Oceanographic Institution placed $5,000 at the disposal of the Library to be used exclusively for the purchase of oceanographic books and journals. Of this amount, $2,149.73 had been spent at the end of the year 1930. The items thus purchased are indicated specifically in the general statement of additions to the Library as follows: journal subscriptions were 346, 24 new, and of these 5 were for the Woods Hole Oceanographic Institution. One hundred and fifteen books were purchased, 45 for oceanography. Back sets of journals were filled in complete to the number of 45, and 37 only partially completed—none of these were regarded as strictly for the Woods Hole Oceanographic Institution. The number of journals received in exchange for the Biological Bulletin was 442, an increase of 22, and 15 back sets that we needed were filled in. The reprint collection was augmented by 5,573.

The Library consists, then, of 26,519 bound journal volumes, 4,991 books, 64,231 reprints; and is receiving 1,060 current journals.

Gifts of books have been made to the Library by the following publishers, and the Librarian takes this opportunity to acknowledge these in the name of the Marine Biological Laboratory Library, although formal thanks have in all cases been directly addressed by letter.

P. Blakiston's Son & Co.	9
R. R. Bowker Co.	1
Chemical Foundation, Inc.	1
Chicago University Press	5
Harvard University Press	2
Paul B. Hoeber	2
Henry Holt & Co.	1
Alfred A. Knopf	2
J. B. Lippincott Co.	1
McGraw-Hill Book Co., Inc.	5
Macmillan Co.	21
C. V. Mosby Co.	1
W. B. Saunders Co.	4
Wm. Wood & Co.	2
Yale University Press	1

MARINE BIOLOGICAL LABORATORY
LIBRARY
WOODS HOLE, MASS.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I beg to submit herewith a report of the forty-third session of the Marine Biological Laboratory for the year 1930.

1. *Attendance.* The attendance for 1930 showed a slight increase over that of the preceding year in the numbers of both investigators and students, the figures for 1930 being 337 investigators and 136 students as compared with 329 investigators and 125 students in 1929. An inspection of the Tabular View of Attendance on page 28 will show that since 1927, when for the first time the research rooms in both the brick and the wooden buildings were practically all in use at the same time, the number of independent investigators has scarcely changed, except for the record-breaking summer of 1929 when visiting foreign physiologists, most of whom came to the Laboratory after the crowded season, swelled the total to figures not likely to be reached under normal conditions. On the other hand, investigators under instruction and research assistants whose numbers are not limited by that of the smaller laboratories have increased from 85 to 120 in the same period. The limit for the further increase of this class of investigators, however, is now in sight, and at the present rate will soon be reached.

The number of students, being limited by action of the Trustees, has shown only minor fluctuations for many years. The slight falling-off in 1929, caused chiefly by the change in that year in the times for holding the courses, was more than made up in 1930, though the maximum number which may at present be admitted to the courses, namely 142, has not yet been reached. This failure of the registration to reach its maximum value is not due to a deficiency of applications, since in nearly all of the courses the number of applicants greatly exceeds the number of available places, but rather to late withdrawals of students who have been accepted. To discourage such withdrawals, which are unfair to the rest of the applicants, who have usually in the meantime made other arrangements for the summer, the Executive Committee has recently voted to make a substantial increase in the registration fee which is forfeited in case of withdrawal.

Following the custom of the past three years, there are here presented figures which show the distribution of the attendance of investigators throughout the four seasons, including that of 1930, for which the necessary records have been kept.

MARINE BIOLOGICAL
WOODS HOLE
LIBRARY
MASS.

		1927	1928	1929	1930
May	30	7	15	9	6
June	10	50	64	55	50
"	20	114	140	139	153
"	30	212	240	197	208
July	10	247	281	238	253
"	20	247	282	242	250
"	30	245	272	249	253
August	10	234	250	256	254
"	20	208	226	243	245
"	30	168	183	220	204
September	10	110	112	157	122
"	20	50	43	59	44
"	30	12	14	14	8

2. *The Report of the Treasurer.* This report shows that the total assets of the Laboratory at the end of 1930 were $2,660,559.11 as compared with $2,660,478.82 at the end of 1929. A further analysis of the figures shows that the book value of the endowment fund has remained practically stationary (though the Treasurer calls attention to a gratifying appreciation in the market value of the securities represented) while additions to the plant assets, chiefly in the form of books and new equipment, have about balanced the estimated depreciation on buildings and equipment. A decrease in the value of the land held by the Laboratory and the simultaneous appearance of a reserve fund of approximately twenty thousand dollars are accounted for by the sale by the Laboratory to the Woods Hole Oceanographic Institution of the tract of land upon which the new building of the latter institution now stands.

The income of the Laboratory increased from $200,408.91 in 1929 to $210,110.86 in 1930. A part of this increase is of a temporary nature only, as for example that from the sale of duplicate sets by the library; of the remainder the largest single item is the additional income from the funds appropriated last year by the General Education Board. In 1930 for the first time the full annual income from this fund became available. Since, however, this fund is at present being applied exclusively to the support of the library, the income available for general laboratory purposes remains practically unchanged.

The ordinary expenses of the Laboratory during 1930 showed a comparatively small increase over those for 1929, but expenses incidental to the reorganization of the Supply Department and the creation of a Museum, and, in particular, the reduction of the inventory of the Supply Department by discarding material originally valued at several thousand dollars, but for which there is at present little or no sale, have again prevented the appearance of a favorable balance, the excess of expenses over income after making allowance for depreciation being $3,767.25 as compared with the similar deficit on paper of $855.33 for 1929. It is evident, however, when account is taken of the unusual expenses of the Supply Department in 1930 and of the necessarily large allowances for depreciation, that the finances of the Laboratory are in a very sound condition.

In 1930, for the first time in many years, the sum paid to the Laboratory for research space, chiefly by subscribing and coöperating institutions, showed a slight decrease. This was to have been expected in view of business conditions, which have materially reduced the incomes of most of the institutions concerned. It is a very encouraging fact, however, that the decrease even under these abnormal conditions amounted to less than three per cent.

3. *The Report of the Librarian* shows a continuation of the steady growth of the library which has been made possible especially by the generous support of the General Education Board. For purposes of comparison the figures for 1930 may be added to those listed in the Director's Report for 1929.

	1925	1926	1927	1928	1929	1930
Serials received currently	500	628	764	874	985	1060
Total number of bound volumes	15000	18200	22800	26500	28300	31500
Reprints	25000	38000	43000	51000	59000	64000

One especially noteworthy feature of the past year has been the strengthening of the part of the library devoted to the subject of oceanography, the development in this direction having been made possible by a coöperative arrangement with the Woods Hole Oceanographic Institution, assisted by special funds appropriated by that institution.

4. *Lectures and Scientific Meetings.* During the season of 1930 the number of formal scientific lectures, including the Reynold A. Spaeth Memorial Lecture delivered by Professor Hardolph Wastenys of the University of Toronto, was thirteen, with several other evenings devoted to non-scientific lectures and motion pictures. In addition, there were held 13 less formal meetings at which 56 shorter papers, whose titles are given on pages 31 to 33, were presented and discussed. Two of these meetings were of especial interest. The first, held on June 27, assumed the character of a celebration of the sixtieth birthday of Dr. Frank R. Lillie and of the fortieth consecutive year of his connection with the Marine Biological Laboratory. In addition to the scientific papers presented on that occason, which were all based upon work having its inception in Dr. Lillie's laboratory, a special address of congratulation was delivered by Dr. E. B. Wilson, and a ship's clock, the gift of Dr. Lillie's former students, was presented to him by Dr. L. V.

Heilbrunn. The evening was concluded by an informal reception at the M. B. L. Club. The second meeting of an unusual character was the one held on the morning of July 26 at which 12 papers in the field of neuro-muscular physiology were presented and discussed. This meeting formed the most important part of a two days' program, social as well as scientific in character, which was arranged by the workers in this field and was attended not only by laboratory workers but by a number of physiologists from a distance. So successful was this meeting that it is to be hoped that similar ones, devoted to various fields of biological research may be held in the future.

5. *Supply Department and Museum.* At the last annual meeting of the Board of Trustees it was voted to develop for the use of investigators and students working in Woods Hole a museum in which specimens of the local fauna and flora may at all times be available for purposes of study, and in which there may at the same time be preserved full records of the distribution of all the local forms, the seasons of their maximum abundance, their breeding habits, etc. The development of this important activity of the Laboratory was very appropriately placed in the hands of Mr. George M. Gray, whose long experience as Curator of the Supply Department has given him unique qualification for such a position. In order to fill the vacancy thus created in the Supply Department and to provide for a possible ultimate separation of the two present functions of this Department, namely, that of supplying living material for experimental purposes to workers at the Laboratory and of furnishing preserved material to schools and colleges, the General Biological Supply House of Chicago was invited to assume its temporary management. In preparation for the new arrangement, Dr. D. L. Gamble, representing this firm, spent several months in residence in Woods Hole during the summer of 1930 and has since continued the general supervision of this Department from Chicago with very satisfactory results, being ably assisted by Mr. James McInnis as Resident Manager.

6. *Facilities for Work with X-rays.* During the summer of 1930, through an appropriation of $500.00 by the Committee on the Effects of Radiation upon Living Organisms of the National Research Council and with the active assistance of the Chairman of that Committee, Professor W. C. Curtis, special facilities, not hitherto available for work with X-rays and other radiations were provided for workers at the Laboratory. In particular, there was made available throughout the summer the expert advice of Dr. G. Failla of the Memorial Hospital, New York, together with the assistance of competent technicians in the operation of the apparatus. Several manufacturers also furnished very valuable aid

of various sorts which is here gratefully acknowledged. So successful was this arrangement in 1930 that it is gratifying to be able to announce at the time of the writing of this report that it will be continued and somewhat extended in 1931.

7. *The Woods Hole Oceanographic Institution.* The position which Woods Hole has held for many years as one of the leading biological centers in the United States was materially strengthened by the erection during the past year of the large and splendidly equipped building which will be the permanent headquarters of the Woods Hole Oceanographic Institution. The land on which this building stands was formerly held by the Marine Biological Laboratory and leased by it to the Bar Neck Corporation. At a special meeting of the Board of Trustees, held in Washington, D. C., on April 28, 1930, it was voted to enter into an agreement with the Woods Hole Oceanographic Institution and the Bar Neck Corporation by which the former institution would acquire by purchase from the Marine Biological Laboratory approximately 54,000 square feet of the westerly portion of the so-called "Bar Neck Wharf." Full details as to this agreement will be found in the Report of the Auditors for 1930. Though there is no official connection between the Woods Hole Oceanographic Institution and the Marine Biological Laboratory, the work of each institution will supplement that of the other, and it is planned that there shall be close scientific coöperation between them. This coöperation has already assumed the form of a sharing of library facilities and Mess accommodations and will be extended in the future in all possible ways.

8. *The Board of Trustees.* One change in the Board of Trustees occurred during the past year, Professor William Patten of Dartmouth College having been elected Trustee Emeritus at the annual meeting of the Corporation and Professor E. R. Clark of the University of Pennsylvania having been selected to fill the place thereby made vacant in the Class of 1934.

9. *Gifts.* Appreciative acknowledgment is made of the gift by Mr. Ware Cattell and the "Collecting Net" of $500.00 for scholarships to students who in the courses given by the Laboratory show unusual promise as research workers.

There are appended as parts of this report:

1. The Staff, 1930.
2. Investigators and Students, 1930.
3. A Tabular View of Attendance, 1926–1930.
4. Subscribing and Coöperating Institutions, 1930.
5. Evening Lectures, 1930.

6. Shorter Scientific Papers, 1930.
7. Members of the Corporation, August, 1930.

Respectfully submitted,

M. H. Jacobs,
Director.

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1. THE STAFF, 1930

Merkel H. Jacobs, *Director,* Professor of General Physiology, University of Pennsylvania.

Associate Director: ——

ZOÖLOGY

I. Investigation

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
E. G. Conklin, Professor of Zoölogy, Princeton University.
Caswell Grave, Professor of Zoölogy, Washington University.
H. S. Jennings, Professor of Zoölogy, Johns Hopkins University.
Frank R. Lillie, Professor of Embryology, University of Chicago.
C. E. McClung, Professor of Zoölogy, University of Pennsylvania.
S. O. Mast, Professor of Zoölogy, Johns Hopkins University.
T. H. Morgan, Director of the Biological Laboratory, California Institute of Technology.
G. H. Parker, Professor of Zoölogy, Harvard University.
E. B. Wilson, Professor of Zoölogy, Columbia University.
Lorande L. Woodruff, Professor of Protozoölogy, Yale University.

II. Instruction

J. A. Dawson, Assistant Professor of Biology, College of the City of New York.
T. H. Bissonnette, Professor of Biology, Trinity College.
E. C. Cole, Associate Professor of Biology, Williams College.
O. E. Nelsen, Instructor in Zoölogy, University of Pennsylvania.
A. W. Pollister, Instructor in Zoölogy, Columbia University.
L. P. Sayles, Instructor in Biology, College of the City of New York.
A. E. Severinghaus, Assistant Professor of Anatomy, College of Physicians and Surgeons, Columbia University.

Junior Instructors

B. R. Coonfield, Professor of Biology, Southwestern College.
I. B. Hansen, Graduate Student, University of Chicago.

PROTOZOÖLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
Rachel Bowling, Instructor in Zoölogy, Columbia University.
Roberts Rugh, Assistant in Zoölogy, Columbia University.

EMBRYOLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

Hubert B. Goodrich, Professor of Biology, Wesleyan University.

Benjamin H. Grave, Professor of Biology, De Pauw University.

Charles Packard, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.

Charles G. Rogers, Professor of Comparative Physiology, Oberlin College.

B. C. Twitty, Instructor in Zoölogy, Yale University.

PHYSIOLOGY

I. Investigation

Harold C. Bradley, Professor of Physiological Chemistry, University of Wisconsin.

Walter E. Garrey, Professor of Physiology, Vanderbilt University Medical School.

Ralph S. Lillie, Professor of General Physiology, University of Chicago.

Albert P. Mathews, Professor of Biochemistry, University of Cincinnati.

II. Instruction

Teaching Staff

William R. Amberson, Assistant Professor of Physiology, University of Pennsylvania.

Philip Bard, Assistant Professor of Physiology, Princeton University.

Hallowell Davis, Assistant Professor of Physiology, Harvard University.

Ralph W. Gerard, Assistant Professor of Physiology, University of Chicago.

Charlotte Haywood, Assistant Professor of Physiology, Vassar College.

Leonor Michaelis, Member of the Rockefeller Institute, New York City.

Special Lecturers

Edwin J. Cohn, Associate Professor of Physical Chemistry, Harvard University.

Henry J. Fry, Associate Professor of Biology, Washington Square College, New York University.

E. Newton Harvey, Professor of Physiology, Princeton University.

Selig Hecht, Professor of Biophysics, Columbia University.

Merkel H. Jacobs, Professor of General Physiology, University of Pennsylvania.

Balduin Lucké, Associate Professor of Pathology, University of Pennsylvania.

BOTANY

I. Investigation

B. M. Duggar, Professor of Physiological and Economic Botany, University of Wisconsin.

C. E. Allen, Professor of Botany, University of Wisconsin.

S. C. Brooks, Professor of Zoölogy, University of California.
Ivey F. Lewis, Professor of Biology, University of Virginia.
Wm. J. Robbins, Professor of Botany, University of Missouri.

II. Instruction

William Randolph Taylor, Professor of Botany, University of Pennsylvania.
H. C. Bold, Instructor in Botany, University of Vermont.
James P. Poole, Professor of Evolution, Dartmouth College.

LIBRARY

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.), Librarian.
Deborah Lawrence, Secretary.
Hester Ann Bradbury, Lillian F. Briggs, Mary A. Rohan, Assistants.

CHEMICAL SUPPLIES

Oliver S. Strong, Professor of Neurology and Neuro-Histology, Columbia University, Chemist.

APPARATUS ROOM

Samuel E. Pond, Assistant Professor of Physiology, Medical School, University of Pennsylvania, Custodian of Apparatus.

MUSEUM

George M. Gray, Curator.

SUPPLY DEPARTMENT

James McInnis, Manager.
A. M. Hilton, Collector.
Milton B. Gray, Collector.
Walter Kahler, Collector.
Geoffrey Lehy, Collector.
A. W. Leathers, Shipping.

BOATS

John J. Veeder, Captain.
E. M. Lewis, Chief Engineer.

F. M. MacNaught, Business Manager.
Herbert A. Hilton, Superintendent of Buildings and Grounds.
Thomas Larkin, Superintendent of Mechanical Department.
Lester F. Boss, Mechanician.
J. D. Graham, Glass-blowing Service.
A. R. Apgar, Photographic Service.
William Hemenway, Carpenter.

2. INVESTIGATORS AND STUDENTS, 1930

Independent Investigators

Abramson, Harold A., Instructor, Harvard University.
Amberson, William R., Assistant Professor of Physiology, University of Pennsylvania.

Armstrong, Philip B., Instructor in Anatomy, Cornell University Medical College.
Ashworth, James H., Professor of Natural History, University of Edinburgh.
Astrom, I. Elisabeth, Class Assistant, University of Toronto.
Baitsell, George A., Professor of Biology, Yale University.
Baker, Horace B., Associate Professor, University of Pennsylvania.
Ball, Eric G., National Research Fellow in Medicine, Johns Hopkins University Medical School.
Bard, Philip, Assistant Professor of Physiology, Princeton University.
Barron, E. S. Guzman, Instructor in Medicine, Johns Hopkins University Medical School.
Barth, L. G., National Research Fellow, University of Chicago.
Beams, H. W., Dupont Fellow, University of Virginia.
Belkin, Morris, Instructor, New York University.
Biddle, Russell L., Teaching Fellow, California Institute of Technology.
Bissonnette, T. Hume, Professor of Biology, Trinity College.
Blanchard, Kenneth C., Associate Professor, New York University.
Blumenthal, Reuben, Harrison Fellow in Zoölogy, University of Pennsylvania.
Bold, Harold C., Instructor in Botany, University of Vermont.
Borodin, D. N., 621 West 142d Street, New York City, New York.
Bowling, Rachel, Instructor in Zoölogy, Columbia University.
Bradway, Winnifred, New York University.
Breitenbecher, J. K., McGill University.
Bridges, Calvin B., Research Assistant, Carnegie Institution of Washington.
Bronfenbrenner, J., Professor of Bacteriology, Washington University Medical School.
Bronk, Detlev W., Professor of Biophysics and Director of Johnson Foundation for Medical Physics, University of Pennsylvania.
Brooks, Matilda M., Research Associate in Biology, University of California.
Brooks, S. C., Professor of Zoölogy, University of California.
Burdick, Donald L., Instructor in Biology, Union College.
Bytinski-Salz, Hans, Research Fellow, Yale University.
Calkins, Gary N., Professor of Protozoölogy, Columbia University.
Carothers, E. Eleanor, Lecturer in Zoölogy, University of Pennsylvania.
Cattell, Ware, Research Fellow in Biophysics, Memorial Hospital.
Chalmers, Elizabeth, Graduate Assistant, University of Pittsburgh.
Chatton, Edouard, University of Strasbourg, Strasbourg, France.
Cheever, Clarence A., Boston Society of Natural History, Boston, Mass.
Chidester, Floyd E., Professor of Zoölogy, West Virginia University.
Chouke, K. S., Assistant Professor of Anatomy, School of Medicine, University of Colorado.
Christie, Jesse R., Associate Nematologist, United States Department of Agriculture.
Clowes, G. H. A., Director, The Lilly Research Laboratory, Eli Lilly & Co.
Cobb, N. A., Agricultural Technologist and Nematologist, United States Department of Agriculture.
Coe, W. R., Professor of Biology, Yale University.
Coffey, J. M., Assistant Bacteriologist, New York State Department of Health.
Cole, Elbert C., Associate Professor of Biology, Williams College.
Cole, Kenneth S., Assistant Professor of Physiology, Columbia University.
Coonfield, Benjamin R., Professor of Biology, Southwestern College.
Cooper, George O., Instructor, University of Wisconsin.
Copeland, Manton, Professor of Biology, Bowdoin College.
Cowles, R. P., Associate Professor of Zoölogy, Johns Hopkins University.
Curtis, W. C., Professor of Zoölogy, University of Missouri.
Davis, Hallowell, Assistant Professor of Physiology, Harvard University Medical School.

Dawson, Alden B., Associate Professor of Zoölogy, Harvard University.
Dawson, J. A., Assistant Professor of Biology, College of the City of New York.
Dill, D. B., Assistant Professor of Biochemistry, Harvard University.
Dolley, William L., Jr., Professor of Biology, University of Buffalo.
Du Bois, Delafield, Washington Square College, New York University.
Du Bois, Eugene F., Associate Professor of Medicine, Cornell University Medical College.
DuBuisson, Marcel, Professor of Zoölogy, "Ecole des Hautes Études," Ghent, Belgium.
Duggar, B. M., Professor of Plant Physiology and Applied Botany, University of Wisconsin.
Edwards, Dayton J., Associate Professor of Physiology, Cornell University Medical College.
Edwards, H. T., Assistant in Fatigue Laboratory, Harvard University.
Failla, G., Physicist, Memorial Hospital, New York.
Fankhauser, Gerhard, Fellow of the Rockefeller Foundation, University of Chicago.
Finley, Harold E., Instructor in Zoölogy, West Virginia State College.
Florkin, Marcel, Research Fellow, Harvard University.
Frew, Priscilla E., Instructor, Hunter College.
Fry, Henry J., Associate Professor of Biology, Washington Square College, New York University.
Furth, Jacob, Associate in Pathology, The Henry Phipps Institute, University of Pennsylvania.
Gardiner, Mary S., Associate in Biology, Bryn Mawr College.
Garrey, W. E., Professor of Physiology, Vanderbilt University Medical School.
Gates, Frederick L., Research Fellow, Harvard University.
Gelfan, Samuel, Research Fellow, University of Chicago.
Gerard, R. W., Assistant Professor of Physiology, University of Chicago.
Gibbons, Norman E., Graduate Student, Yale University.
Goldforb, A. J., Professor of Biology, College of the City of New York.
Goodrich, Hubert B., Professor of Biology, Wesleyan University.
Grave, B. H., Professor of Zoölogy, DePauw University.
Grave, Caswell, Professor of Zoölogy, Washington University.
Grundfest, Harry, National Research Council Fellow, Columbia University.
Hance, Robert T., Head of Department of Zoölogy, University of Pittsburgh.
Harvey, Ethel B., Instructor, Washington Square College, New York University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Haywood, Charlotte, Assistant Professor of Physiology, Vassar College.
Heilbrunn, L. V., Associate Professor of Zoölogy, University of Pennsylvania.
Henshaw, Paul S., Biophysicist, Memorial Hospital.
Hetler, Donald M., Instructor in Bacteriology, Washington University Medical School.
Hibbard, Hope, Assistant Professor, Oberlin College.
Hill, Samuel E., Assistant in Physiology, Rockefeller Institute.
Hinrichs, Marie A., Research Associate in Physiology, University of Chicago.
Hintze, A. Laura, Assistant Professor of Physiology, Goucher College.
Hirsch, G. C., Professor of Zoölogy, University of Utrecht.
Hoppe, Ella N., Research Assistant, New York State Department of Health.
Howe, H. E., Editor, Industrial and Engineering Chemistry.
Howland, Ruth B., Associate Professor of Biology, Washington Square College, New York University.
Huettner, Alfred F., Associate Professor, Washington Square College, New York University.
Hughes, Thomas P., Associate in Bacteriology, Rockefeller Institute.
Hunter, Lillian M., Graduate Student and Assistant Technician, University of Toronto.

Hutchinson, G. E., Instructor in Biology, Yale University.
Hyman, Libbie H., Research Associate, University of Chicago.
Jacobs, M. H., Professor of General Physiology, University of Pennsylvania.
Jennings, H. S., Professor of Zoölogy, Johns Hopkins University.
Johlin, J. M., Associate Professor of Biochemistry, Vanderbilt University Medical School.
Johnson, H. Herbert, Instructor, College of the City of New York.
Just, E. E., Professor of Zoölogy, Howard University.
Keil, Elsa M., Instructor in Zoölogy, Rutgers University.
Keltch, Anna K., Research Chemist, Lilly Research Laboratory.
Kettlekamp, B. H., Instructor, University of Pittsburgh.
Keyes, D. B., Professor of Industrial Chemistry, University of Illinois.
Keys, Ancel B., Fellow in the Biological Sciences, National Research Council.
Knower, Henry McE., Wistar Institute.
Knowlton, Frank P., Professor of Physiology, Syracuse University, College of Medicine.
Koehring, Vera, Beaver College, Jenkintown, Pennsylvania.
Kunitz, Moses, Associate Professor, Rockefeller Institute for Medical Research.
Lackey, J. B., Professor of Biology, Southwestern University.
Lancefield, D. E., Associate Professor in Zoölogy, Columbia University.
Lancefield, Rebecca C., Assistant in Bacteriology, Rockefeller Institute for Medical Research.
Lewis, Ivey F., Professor of Biology, University of Virginia.
Lillie, Frank R., Chairman of the Department of Zoölogy, University of Chicago.
Lillie, Ralph S., Professor of General Physiology, University of Chicago.
Lucké, Balduin, Associate Professor of Pathology, University of Pennsylvania.
Lynch, Ruth S., Instructor in Graduate Zoölogy, The Johns Hopkins University.
Lyon, E. P., Professor of Physics, University of Minnesota.
McClung, C. E., Director, Zoölogical Laboratory, and Professor of Zoölogy, University of Pennsylvania.
MacDougall, Mary S., Head of Biology Department, Agnes Scott College.
McEwen, Robert S., Associate Professor of Zoölogy, Oberlin College.
McGlone, Bartgis, Instructor in Physiology, University of Pennsylvania.
Manwell, Reginald D., Instructor, Johns Hopkins University.
Marsland, Douglas A., Assistant Professor of Biology, Washington Square College, New York University.
Mathews, A. P., Professor of Biochemistry, University of Cincinnati.
Matthews, Samuel A., Instructor, University of Pennsylvania.
Mavor, James W., Professor of Biology and Head of Department, Union College.
Menkin, Valy, Fellow in Medicine, Henry Phipps Institute, University of Pennsylvania.
Meyer, Roland K., Research Assistant, University of Wisconsin.
Michaelis, Leonor, Member, Rockefeller Institute for Medical Research.
Miller, Helen M., Fellow, National Research Council, Johns Hopkins University.
Mitchell, Philip H., Professor of Physiology, Brown University.
Monnier, Andreé, University of Paris.
Monnier, A. M., Assistant at the Sorbonne, Paris.
Morgan, Lilian V., California Institute of Technology.
Morgan, T. H., Director of the Biological Laboratory, California Institute of Technology.
Morgulis, S., Professor of Biochemistry, University of Nebraska, College of Medicine.
Morrill, C. V., Associate Professor of Anatomy, Cornell University Medical College.
Morris, Helen S., Graduate Student, Columbia University.
Mulder, Arthur G., Associate Professor of Physiology, University of Tennessee.

Nabrit, S. Milton, Head of Department of Biology, Morehouse College.
Nelson, Olin E., Instructor in Zoölogy, University of Pennsylvania.
Nicholas, Warren W., X-Ray Physicist, National Bureau of Standards.
Nonidez, José F., Assistant Professor of Anatomy, Cornell University Medical College.
Packard, Charles, Assistant Professor of Zoölogy, Columbia University.
Park, Orlando, Associate Professor of Biology, Kent State College.
Parmenter, Charles L., Associate Professor of Zoölogy, University of Pennsylvania.
Payne, Fernandus, Professor of Zoölogy and Dean of Graduate School, Indiana University.
Perrot, J. L., Columbia University.
Petrik, Joseph M., Director of the Department of Physiology, Masaryk University.
Phillips, Paul L., Instructor in Anatomy, Cornell University Medical College.
Philpott, Charles H., Lecturer in Medical Zoölogy, Washington University Medical School.
Plough, Harold H., Professor of Biology, Amherst College.
Pollister, Arthur W., Instructor in Zoölogy, Columbia University.
Pond, Samuel E., Assistant Professor of Physiology, University of Pennsylvania Medical School.
Poole, James P., Professor of Evolution, Dartmouth College.
Potter, Truman S., Seymour Coman Fellow, University of Chicago.
Raffel, Daniel, National Research Fellow, Johns Hopkins University.
Redfield, Helen, California Institute of Technology.
Reese, Albert M., Head of Department of Zoölogy, West Virginia University.
Richards, Oscar W., Assistant Professor of Biology, Clark University.
Roberts, Edith A., Chairman of Department of Botany, Vassar College.
Rogers, Charles G., Professor of Comparative Physiology, Oberlin College.
Romanoff, Alexis L., Research Instructor, Cornell University.
Root, Walter S., Assistant Professor, Syracuse University Medical School.
Rugh, Roberts, Assistant in Zoölogy, Columbia University.
Sayles, Leonard P., Instructor in Zoölogy, Tufts College.
Schauffler, William G., Princeton, New Jersey.
Schmidt, Leon H., University of Cincinnati.
Schmitt, Francis O., Assistant Professor of Zoölogy, Washington University.
Schrader, Franz, Associate Professor, Bryn Mawr College.
Schrader, Sally-Hughes, Instructor in Biology, Bryn Mawr College.
Schultz, Jack, Research Assistant, Carnegie Institution of Washington.
Severinghaus, Aura E., Associate in Anatomy, Medical School, Columbia University.
Sharma, B. M., Professor of Anatomy, Tibbi Medical College.
Shoup, Charles S., Assistant Professor of Biology, Vanderbilt University.
Shull, A. Franklin, Professor of Zoölogy, University of Michigan.
Shumway, Waldo, Professor of Zoölogy, University of Illinois.
Sichel, Ferdinand J., Assistant Instructor, Washington Square College, New York University.
Slifer, Eleanor H., Graduate Student, University of Pennsylvania.
Smith, Frederick, Research Assistant, Rockefeller Institute.
Steinbach, H. B., Graduate Student, Brown University.
Stockard, Charles R., Professor of Anatomy, Cornell University Medical College.
Strong, Oliver S., Professor of Neurology and Neuro-Histology, Columbia University.
Stunkard, H. W., Professor of Biology, New York University.
Sturdivant, Harwell P., Instructor, Columbia University.
Sturtevant, A. H., Professor of Genetics, California Institute of Technology.

SUMWALT, MARGARET, Assistant Professor, Woman's Medical College.
TASHIRO, SHIRO, Professor of Biochemistry, The University of Cincinnati.
TAYLOR, WM. RANDOLPH, Professor of Botany, University of Pennsylvania.
THOMPSON, J. W., Instructor, Swarthmore College.
TOWER, SARAH S., Instructor in Anatomy, Johns Hopkins University.
TWITTY, VICTOR C., Instructor in Zoölogy, Yale University.
UHLENHUTH, EDUARD, Professor of Gross Anatomy, University of Maryland Medical School.
VAN CLEAVE, C. D., Instructor in Anatomy, University of Pennsylvania.
VAN SLYKE, E., Instructor, University of Pittsburgh.
WANG, CHI CHE, 1314 East 56th Street, Chicago, Illinois.
WARREN, HOWARD C., Stuart Professor of Psychology, Princeton University.
WENRICH, D. H., Professor of Zoölogy, University of Pennsylvania.
WESSON, LAURENCE G., Assistant Professor of Pharmacology, Vanderbilt University Medical School.
WHEDON, ARTHUR D., Professor of Zoölogy and Head of Department, North Dakota Agricultural College.
WHITAKER, D. M., Assistant Professor of Zoölogy, Columbia University.
WHITING, ANNA R., Professor, Head of Department of Biology, Pennsylvania College for Women.
WHITING, P. W., Associate Professor of Zoölogy, University of Pittsburgh.
WIEMAN, H. L., Professor of Zoölogy, University of Cincinnati.
WILLIER, B. H., Associate Professor of Zoölogy, University of Chicago.
WILSON, EDMUND B., DaCosta Professor of Zoölogy, Emeritus, Columbia University.
WILSON, F. EDWARD, Graduate Student, Clark University.
WILSON, MAY G., Associate, Department of Pediatrics, Cornell University Medical College.
WOLF, E. ALFRED, Assistant Professor of Zoölogy, University of Pittsburgh.
WOODRUFF, L. L., Professor of Protozoölogy, Yale University.
WOODS, FARRIS H., Assistant Professor of Zoölogy, University of Missouri.
YOUNG, WILLIAM C., Instructor in Biology, Brown University.
ZELENY, CHARLES, Professor of Zoölogy, University of Illinois.

Beginning Investigators

BAILEY, SARAH W., Graduate Student, Radcliffe College.
BROWN, FRANK A., JR., Austin Teaching Fellow, Harvard University.
CALDWELL, LUCILE, Johns Hopkins University.
CHANG, J. H., Graduate Student, University of Chicago.
CHEN, H. T., Student, Harvard University Medical School.
CLINE, ELSIE, Graduate Student, The Johns Hopkins University.
COLDWATER, KENNETH B., Instructor in Zoölogy, University of Missouri.
COSTELLO, DONALD P., Assistant in Zoölogy, College of the City of Detroit.
CROASDALE, HANNAH T., Graduate Student, University of Pennsylvania.
CURTIS, MARY ELIZABETH, Graduate Student, Yale University.
DAMBACH, GEORGE J., Graduate Assistant, University of Pittsburgh.
DEARING, WILLIAM H., Graduate Student, University of Pennsylvania.
DEITRICH, JOHN E., Medical Student, Johns Hopkins University.
DIXON, EVELYN C., Graduate Student, Washington University.
DRAPER, JOHN W., Cornell University Medical College.
DREW, WILLIAM, Massachusetts Agricultural College.
DUSHANE, GRAHAM, Wabash College.
ETKIN, WILLIAM, Tutor, College of the City of New York.
FLAMMON, SISTER M. MURIEL, Instructor in Biology, Seton Hill College.
FRENCH, C. S., Harvard University.
GEBRAK, ANTON, Moscow Agricultural Academy.

Geib, Dorothy A., Student, Johns Hopkins University Medical School.
Geiman, Quentin M., Graduate Student, University of Pennsylvania.
Genther, Ida T., Assistant in Zoölogy, Washington University.
Graubard, Marc A., Assistant in Zoölogy, Columbia University.
Green, David E., Assistant, New York University.
Guerlac, Henry E., Cornell University.
Hansen, Ira B., Instructor, Wesleyan University.
Haynes, Florence W., 20 Gorham Road, West Medford, Massachusetts.
Hegnauer, Albert, Assistant in Physiology, University of Rochester, School of Medicine.
Hileman, Clara M., Teacher of Biology, Columbia University.
Hoerr, Stanley O., Antioch College.
Hook, Sabra J., Assistant in Zoölogy, Barnard College, Columbia University.
Iglauer, Charles A., Graduate Student, University of Pennsylvania.
Imlah, Helen W., Graduate Student, Radcliffe College.
Jeffery, Helen, Research, Washington University Medical School.
Kaltreider, Nolan L., Student, Johnson Foundation, University of Pennsylvania.
Katz, Jacob D., Assistant Instructor, Washington Square College, New York University.
Kerr, Thomas, Instructor in Biology, New York University.
Kinney, Elizabeth T., Assistant at Barnard College, Columbia University.
Kinsbergen, Maurice, Assistant, New York University.
Klose, Theodora, Instructor in Botany, Vassar College.
Lee, Katy, Graduate Assistant in Zoölogy, University of Missouri.
Lhérisson, Camille, Professor of Biology, University of Haiti Medical School.
Lorberblatt, Isaac, Student, Washington University Medical School.
McGoun, Ralph C., Jr., Instructor in Biology, Amherst College.
MacMurray, Mary T., 8629 109th Street, Richmond Hill, New York.
Mackmull, Gulden, Demonstrator of Histology and Embryology, Baugh Institute of Anatomy.
Miller, Evelyn H., Graduate Student, University of Pennsylvania.
Miller, Forrest W., Graduate Assistant, University of Pittsburgh.
Monaghan, Betty R., Assistant, Washington University.
Morris, Samuel, Instructor in Zoölogy, University of Pennsylvania.
Nelson, Phyllis M., Washington University.
Parpart, Arthur K., Instructor and Graduate Student, University of Pennsylvania.
Parpart, Ethel R., Technician, University of Pennsylvania.
Pitts, Robert F., Student Assistant, Johns Hopkins University.
Rankin, Douglas, Johns Hopkins University.
Robert, Nan L., Instructor, Hunter College.
Santos, Felix V., Graduate Student, The University of Chicago.
Savin, Marion B., Graduate Student, University of Pennsylvania.
Scarborough, J. Elliott, Jr., Student of Medicine, Harvard University.
Schechter, Victor, Tutor in the Department of Biology, College of the City of New York.
Schweitzer, Morton D., Assistant in Zoölogy, Columbia University.
Scott, Sister Florence M., Assistant Professor of Biology, Seton Hill College.
Shapiro, Herbert, Assistant in Zoölogy, Columbia University.
Shaw, C. Ruth, Graduate Assistant, University of Pittsburgh.
Skinner, B. F., Graduate Student, Harvard University.
Smith, Suzanne G., Graduate Assistant in Zoölogy, University of Missouri.
Smythe, C. V., Fellow National Research Council, Rockefeller Institute.
Sonneborn, Tracy M., Research Assistant, Johns Hopkins University.
Stabler, Robert M., Assistant Instructor, University of Pennsylvania.
Stancati, Milton F., Graduate Assistant, University of Pittsburgh.
Stewart, Dorothy R., Assistant Professor of Biology, Skidmore College.

Steiner, Matthew M., Assistant in Biology, New York University.
Street, Sibyl F., Assistant to Department of Zoölogy, Vassar College.
Stuck, Florence, Student, Columbia University.
Tang, Pei-sung, Johns Hopkins University.
TeWinkel, Lois E., Assistant in Zoölogy, Barnard College, Columbia University.
Toothill, Martha C., Assistant in Biology, Brown University.
Tuan, Hsu-Chuan, Graduate Student, University of Pennsylvania.
Turner, Clarence D., Research Assistant, University of Missouri.
Waters, Nelson F., Research Assistant in Applied Botany, Harvard University.
Wells, Evelyn, Instructor in Biology, St. Mary's Seminary.
Wells, L. J., Graduate Student, University of Chicago.
Wilde, Mary H., Graduate Assistant, New Jersey College for Women.
Wright, Charles I., Fellow in Physiology, University of Rochester Medical School.
Yancey, Patrick H., Graduate Student, St. Louis University.

Research Assistants

Beck, L. V., Teaching Fellow, New York University.
Bernstein, Alan, Research Assistant, New York University.
Buck, Louise H., Research Assistant, New York University.
Campbell, Raymond W., Assistant, Fatigue Laboratory, Harvard University.
Davidson, Sydney A., Williams College.
Eisenbrandt, W. H., Student, University of Maryland Medical School.
Francis, Dorothy S., Research Assistant, Memorial Hospital.
Friedman, Hilda, Assistant in Pathology, Washington University Medical School.
Friedheim, Ernst A. H., Rockefeller Institute.
Grand, Constantine G., Research Assistant, New York University.
Greenberg, Jacob, Medical Student, Yale University Medical School.
Harryman, Ilene, Research Assistant, Lilly Research Laboratory.
Heusner, A. P., Student, Swarthmore College.
Hoffman, Olive D., Research Assistant, New York University.
Lazarovich-Hrebelianovich, Mara de, Research Assistant, New York University.
Mendelson, E. S., Research Assistant, University of Pennsylvania.
Menkin, Miriam F., Henry Phipps Institute.
Oberg, S. Albert, Harvard University.
Parks, Elizabeth K., Graduate Assistant in Zoölogy, Oberlin College.
Ravenswaay, van A. C., Research Assistant, Washington University.
Reynolds, Sara Jane, Research Assistant, New York University.
Rudnick, Dorothea, Research Assistant, University of Chicago.
Salomon, Kurt, Fellow of the Rockefeller Foundation, Rockefeller Institute for Medical Research.
Sanders, Rosaltha, Technician, Yale University.
Schubert, Maxwell, Assistant, Rockefeller Institute for Medical Research.
Scott, Allan C., Graduate Assistant, University of Pittsburgh.
Shattuck, G. Edgar, Assistant Instructor in Physiology, University of Pennsylvania.
Shear, M. J., Administrative Officer and Research Chemist, Pediatric Research Laboratory, Jewish Hospital.
Shlaer, Simon, Research Assistant, Columbia University.
Smith, M. Doreen, Research Assistant, Memorial Hospital.
Swann, Sherlock, Research Associate, University of Illinois.
Tocker, Albert M., Student, Washington University Medical School.
Wallace, Edith M., Artist and Research Assistant, Carnegie Institution of Washington.

Students

BOTANY

Breed, Helen L., Student, Wellesley College.
Brunel, Jules, Assistant Professor of Botany, University of Montreal.
Cheever, Clarence A., Boston Society of Natural History.
Drouet, Francis, Graduate Assistant, University of Missouri.
Forbes, John M., Student, Harvard University.
Glidden, Dorothy P., Student, Smith College.
Hopkins, Milton, Student, Amherst College.
Huntington, Evelyn, Student, Vassar College.
Klose, Theodore G., Instructor in Botany, Vassar College.
Loughridge, Gasper A., Laboratory Assistant in Botany, Rutgers University.
McKee, Jewel C., University of Wisconsin.
Oppenheimer, Jane M., Student, Bryn Mawr College.
Roland, Albert E., Student, Acadia University.
Safford, Decius W., Dartmouth College.
Stewart, Paul A., Student, University of Rochester.
Wilde, Mary H., Assistant, New Jersey College for Women.

EMBRYOLOGY

Baker, E. G. Stanley, Student, DePauw University.
Ballard, Overton T., University of Illinois.
Bamber, Lyle E., Graduate Student and Assistant, University of Illinois.
Carter, George H., Student, Amherst College.
Dawson, Helen L., Graduate Student, Washington University.
Derbyshire, Arthur J., Jr., Harvard University.
Earl, Ruth R., Technician, College of the City of New York.
Eaton, Theodore H., Jr., Cornell University.
Green, David E., Assistant, Washington Square College.
Guerlac, Henry E., Student, Cornell University.
Hileman, Clara M., Instructor in Biology, Penn State College.
Hjortland, Arthur L., Assistant, University of Illinois.
Hunninen, Arne V., Student, Wesleyan University.
Johnson, Arlene C., Student, Wheaton College.
Johnson, Myra L., Student, Smith College.
Lane, Mary, Smith College.
Loefer, John B., Graduate Assistant in Biology, New York University.
Mackmull, Gulden, Demonstrator of Histology and Embryology, Baugh Institute of Anatomy, Jefferson Medical College.
Maxwell, Florence J., Instructor, Carnegie Institute of Technology.
Nichols, Rowena, Wellesley College.
Patch, Esther M., Teacher of Biology, East Boston High School.
Reid, Marion A., Instructor, Boston University Medical School.
Riley, Lena C., Student, Wellesley College.
Rosenbaum, Louise, Student, University of Pennsylvania.
Scott, John P., University of Wyoming.
Stanley, Willard F., Graduate Student and Research Assistant, University of Illinois.
Wells, Lemen J., Graduate Student, University of Chicago.

PHYSIOLOGY

Appelrot, Samuel, Fellow, Rockefeller Foundation.
Behner, Dorothy M., Assistant, New York University.
Chang, Tsung H., Graduate Student, University of Chicag .

Chen, Tuang T., Assistant in Biochemistry, Peking Union Medical College.
Dann, Margaret, Assistant in Physiology, Cornell University Medical College.
Drew, William B., Massachusetts Agricultural College.
DuBois, Delafield, New York University.
Feng, Te-Pei, Graduate Student, University of Chicago.
Gardner, Edith McN., Assistant in Physiology, Vassar College.
Gates, Frederick L., Research Fellow, Harvard University.
Hegnauer, Albert H., Fellow in Physiology, University of Rochester Medical School.
Leitch, James L., University of California.
Monaghan, Betty R., Assistant and Graduate Student, Washington University.
Oliphant, Joseph F., Instructor in Biology, Union College.
Oster, Robert H., Student, Williams College.
Pitts, Robert F., Johns Hopkins University.
Shaw, Gretchen, Graduate Student, University of Chicago.
Steiner, Matthew M., Assistant in Biology, New York University.
Stevens, Thelma O., Graduate Assistant, Mt. Holyoke College.
Tang, Pei-sung, Harvard University.
Vack, Christine M., Technician, Harvard University Medical School.
Vicari, Emilia M., Research Associate, Cornell University Medical College.
Woodward, Alvalyn E., Assistant Professor, University of Michigan.

PROTOZOÖLOGY

Brehme, Katherine S., Barnard College.
Carter, Helen D., Elmira College.
Costello, Donald P., 3732 Heidelberg, Detroit, Michigan.
Embich, John R., Graduate Student, Columbia University.
Flammon, Sister M. Muriel, Seton Hill College.
Frye, Mary Elizabeth, Pennsylvania College for Women.
Lhérisson, Camille, Professor of Biology, University of Haiti Medical School.
Masten, Lois E., Elmira College.
Morgan, Willie A., Assistant Instructor in Biology, Coker College.
Schoelt, Abraham H., Graduate Student, Columbia University.
Scotland, Minnie B., Teacher, New York State College for Teachers.
Smith, Claire M., Hunter College.
Steinberg, Bernhard, Director of Laboratories and Research, Toledo Hospital.
Weisman, Maxwell N., Fellow, College of the City of New York.

INVERTEBRATE ZOÖLOGY

Alderman, Evangeline, Oberlin College.
Baumgartner, Frederick M., Butler University.
Bittinger, Isabel, Radcliffe College.
Boardman, Edward T., Graduate Assistant, Johns Hopkins University.
Brown, Frank A., Jr., Harvard University.
Campbell, Dan H., Student, Wabash College.
Carlson, J. Gordon, Assistant in Zoölogy, University of Pennsylvania.
Chadwick, Claude S., Instructor in Biology, Vanderbilt University.
Cohen, Rose S., Graduate Assistant, University of Cincinnati.
Coleman, Lucille, Agnes Scott College.
Coplan, Helen M., Student, Goucher College.
Craig, Robert L., Student, Amherst College.
Crowell, Prince S., Jr., Bowdoin College.
Dee, M. Barbara, Assistant in Science, Jamaica Plain High School.
DeRoo, Grace, Radcliffe College.
Derrickson, Mary B., Goucher College.

Dorris, Frances S., Graduate Student, Yale University.
Doyle, William L., Johns Hopkins University.
Eichold, Eva C., Student, Newcomb College.
Everett, John W., Yale University.
Farber, Seymour M., University of Buffalo.
Fennell, R. A., Graduate Student, Duke University.
Fisher, Kenneth C., Student and Assistant, Acadia University.
French, Charles S., Harvard University.
Hamburger, Louis P., Jr., Johns Hopkins University.
Hart, Helen B., Student, Wellesley College.
Hastings, Margaret, Student, Mt. Holyoke College.
Hayes, Frederick R., Assistant Professor of Zoölogy, Dalhousie University.
Heiss, Elizabeth M., Assistant in Biology and Histology, Purdue University.
Heusner, Albert P., Swarthmore College.
Hewitt, Dorothy C., Graduate Student, Yale University.
Holloway, May P., Teacher of Science, Burke School.
Hubbard, Ruth A., Assistant, Cleveland Museum of Natural History.
Jackson, Jeannette A., Graduate Assistant in Zoölogy, Syracuse University.
Johnson, Dorothy F., Laboratory Assistant, Wellesley College.
Kille, Frank R., Assistant Professor of Biology, Birmingham-Southern College.
Kroc, Robert L., Graduate Assistant, University of Wisconsin.
Leavitt, Benjamin B., Instructor in Zoölogy, Dartmouth College.
Menzel, Arthur E. O., National Tuberculosis Research Fellow, Presbyterian Hospital.
Merriman, Daniel, Student, Harvard University.
Morris, Samuel, Instructor in Zoölogy, University of Pennsylvania.
Prest, Margaret R., Graduate Assistant, Mt. Holyoke College.
Redmond, Albert C., Student, Hamilton College.
Reynolds, Albert E., Assistant in Zoölogy, DePauw University.
Ritter, M. Esther, Student, Wilson College.
Shea, Margaret, Student, Oberlin College.
Sidebotham, Ruth S., Graduate Assistant in Zoölogy, Washington University.
Snell, Peter A., Fellow in Biology, Princeton University.
Snook, Theodore, Graduate Assistant in Zoölogy, Rutgers University.
Swanson, Oscar E., Student, Antioch College.
Tipton, Samuel R., Graduate Student, Duke University.
Townsend, Grace, Instructor, Joliet Township High School and Junior College.
Treat, Dorothy A., Assistant in Department of Education, Cleveland Museum of Natural History.
Waters, Nelson F., Graduate Student, Harvard University.
Weed, Milton R., Student, Wesleyan University.
Woodruff, Beth H., Graduate Assistant, Western Reserve University.

3. TABULAR VIEW OF ATTENDANCE

	1926	1927	1928	1929	1930
INVESTIGATORS—Total	252	294	323	329	337
Independent	156	209	217	234	217
Under Instruction	84	57	81	71	87
Research Assistants	12	28	25	24	33
STUDENTS—Total	141	141	133	125	136
Zoölogy	56	57	57	53	56
Protozoölogy	19	17	16	15	14
Embryology	28	32	29	28	27
Physiology	18	19	15	17	23
Botany	20	16	16	12	16
TOTAL ATTENDANCE	393	435	456	454	473
Less Persons registered as both students and investigators	8	1	2	10	14
	385	434	454	444	459
INSTITUTIONS REPRESENTED—Total	119	111	111	123	126
By Investigators	84	89	80	96	95
By Students	60	63	66	64	71
SCHOOLS AND ACADEMIES REPRESENTED					
By Investigators	–	1	1	–	–
By Students	4	4	1	1	4
FOREIGN INSTITUTIONS REPRESENTED					
By Investigators	17	15	13	30	7
By Students	3	8	8	3	2

4. SUBSCRIBING AND COÖPERATING INSTITUTIONS

Acadia University
Amherst College
Antioch College
Beaver College
Bowdoin College
Brown University
Bryn Mawr College
Butler College
C. R. B. Educational Foundation
California Institute of Technology
Carnegie Institution of Washington
Chinese Educational Mission
Columbia University
Cornell University
Cornell University Medical College
Dalhousie University
Dartmouth College
DePauw University
Duke University
Elmira College
General Education Board
Goucher College
Hamilton College
Harvard University
Harvard University Medical School
Howard University
Hunter College
Indiana University
Industrial & Engineering Chemistry, of the American Chemical Society
Johns Hopkins University
Johns Hopkins University Medical School
Eli Lilly & Co.
Memorial Hospital of N. Y. City
Morehouse College
Mount Holyoke College
National Research Council
New York State Department of Health
New York University
Oberlin College

Pennsylvania College for Women
Princeton University
Radcliffe College
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
Seton Hill College
Smith College
Sophie Newcomb College
Southwestern
St. Louis University
Swarthmore College
Tufts College
Union College
United States Dept. of Agriculture
University of Buffalo
University of Chicago
University of Cincinnati
University of Illinois
University of Michigan
University of Missouri
University of Nebraska
University of Pennsylvania
University of Pennsylvania Medical School
University of Pittsburgh
University of Rochester
University of Tennessee
University of Virginia
University of Wisconsin
Vanderbilt University Medical School
Vassar College
Wabash College
Washington University
Washington University Medical School
Wellesley College
Wesleyan University
Western Reserve University
West Virginia State College
West Virginia University
Wheaton College
Wistar Institute of Anatomy and Biology
Yale University

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SCHOLARSHIP TABLES

Lucretia Crocker Scholarships for Teachers in Boston.
Scholarship of $100 supported by a friend of the Laboratory since 1898.
The Edwin S. Linton Memorial Scholarship of Washington and Jefferson College.
The Bio Club Scholarship of the College of the City of New York.
Ida H. Hyde Scholarship of the University of Kansas.

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5. EVENING LECTURES, 1930

Tuesday, July 1
Dr. W. B. Scott .... "New Light on the Development and Migrations of American Mammals."

Tuesday, July 8
Dr. Henry B. Bigelow .... "Prospects and Problems of Oceanography."

Tuesday, July 15
Dr. H. S. Jennings .... "Heredity and Mutation in Relation to Environment in Protozoa."

Friday, July 18
Dr. Edward Mellanby .... "Food Deficiencies."

Tuesday, July 22
Dr. F. R. Lillie .... "The Action of the Sex Hormones in the Fowl: An Account of the Chicago Experiments."

Tuesday, July 29
Dr. Leonor Michaelis .... "The Reversible Oxidizable-reducible Systems Found in Living Organisms."

Tuesday, August 5
Dr. E. F. DuBois .... "Recent Progress in the Field of Respiratory Metabolism."

Tuesday, August 12
Dr. E. Chatton .... "A Study of the Dinoflagellate, Polykrikos Schwartzi as a Basis for the Discussion of Some Problems of General Cytology."

Tuesday, August 19
The Reynold A. Spaeth Memorial Lecture, delivered by Dr. Hardolph Wasteneys .... "Protein Synthesis."

Thursday, August 21
Dr. H. H. Gran .... "The Productivity of the Ocean."

Tuesday, August 26
Dr. D. W. Bronk .... "Nerve Impulse Rhythms and the Control of Movement."

Tuesday, September 2
Dr. G. C. Hirsch .... "The Problem of Restitution with Special Regard to the Phenomena of Secretion."

Tuesday, September 9
Dr. G. E. Hutchinson .... "The Hydrobiology of Arid and Semi-arid Regions."

SPECIAL LECTURES AND MOTION PICTURES

Monday, August 11

"The Illyria Expedition to the Galapagos, the South Sea Islands, the New Hebrides, the Solomon Islands, New Guinea, Bali and Angkor." Exhibited by Mr. Cornelius Crane, the Leader of the Expedition.

Thursday, August 14

"The Florida Everglades and the Proposed Tropic Everglades National Park." Illustrated with colored lantern slides. Mr. Ernest F. Coe, Chairman of the Tropic Everglades Park Association.

Thursday, August 28

Motion pictures.

"William Harvey and the Circulation of the Blood." Arranged by Sir Thomas Lewis.

"The Early Development of the Rabbit Egg." Dr. W. H. Lewis and Dr. P. W. Gregory.

"The Life Cycle of the Oyster." Prepared by the American Museum of Natural History.

Friday, August 29

"Motion Pictures of Marine and Fresh Water Protozoa of the Woods Hole Region," Ruth B. Howland, Department of Biology, New York University.

6. SHORTER SCIENTIFIC PAPERS, 1930

Tuesday, June 24	
Dr. Balduin Lucké and Dr. Morton McCutcheon	"The Effect of Injury on Cellular Permeability to Water."
Dr. M. M. Brooks	"The Relation between rH and the Penetration of Oxidation-reduction Indicators."
Dr. S. C. Brooks	"Accumulation of Ions in Living Cells."
Dr. M. H. Jacobs, Mr. A. K. Parpart, Dr. W. A. Smith and Mr. G. E. Shattuck	"The Permeability of the Erythrocyte to Urea."
Friday, June 27	
Dr. B. H. Willier	"The Developmental Relations of the Heart and the Liver in Chorio-allantoic Grafts."
Dr. E. E. Just	"Cortical Protoplasm and Vital Phenomena."
Dr. William C. Young	"The Post-testicular History of Spermatozoa and Reproduction in the Male Guinea Pig."
Dr. L. V. Heilbrunn	"The Action of Ultra-violet Rays on Arbacia Egg Protoplasm."
Thursday, July 3	
Dr. Walter S. Root and Dr. Charlotte Haywood	"The Effect of Carbon Dioxide upon the Rate of Oxygen Consumption and of Cleavage of the Arbacia Egg."
Dr. L. Michaelis and Dr. K. Salomon	"Respiration of Erythrocytes."
Dr. R. W. Gerard	"Observations on the Metabolism of the Coccus, Sarcina lutea."
Dr. E. S. G. Barron	"The Effect of Methylene Blue upon the Respiration of Normal and of Cancer Tissue."
Friday, July 11	
Dr. W. R. Taylor	"Chromosome Structure in Meiosis of Gasteria."
Dr. B. M. Duggar	"New Technique and Some Adsorption Studies with Virus Diseases of Plants."
Thursday, July 17	
Dr. Vera Koehring	"Some Cytological Relationships in Narcosis."
Dr. Eleanor H. Slifer	"The Mitotic Activity in the Developing Grasshopper Egg."

Dr. A. F. Huettner	"Spermatogenesis in Drosophila melanogaster."
Dr. Hope Hibbard	"Cytological Studies on the Silk Gland of Bombyx mori."
Friday, July 25	
Dr. S. Gelfan	"The All-or-None Law in Muscle."
Dr. W. R. Amberson, Mr. A. K. Parpart and Miss Gertrude Sanders	"Low Voltage Elements of the Action Potential Wave of Nerve."
Dr. F. O. Schmitt	"The Effect of Cyanides and Carbon Monoxide on Nerve."
Saturday, July 26	
Dr. M. Dubuisson	"Cardiac Automatism in Invertebrates."
Dr. W. E. Garrey	"Observations on the Heart of Limulus."
Dr. D. J. Edwards	"The Action of Pressure on Some Physiological Processes."
Dr. G. H. Bishop	"The Influence of Iodo-acetic Acid on Muscle Contracture."
Dr. F. H. Pratt	"Experiments on the Terminal Nerve-muscle Unit."
Dr. D. W. Bronk	"Graded Muscular Contractions."
Dr. R. W. Gerard	"Nerve Metabolism and Asphyxia."
Dr. R. S. Lillie	"Recovery in the Passive Iron Wire Model."
Dr. A. M. Monnier	"Mathematical Analysis Applied to the Functions of the Nervous System."
Dr. H. Davis	"Re-education and Modification of Reflexes."
Dr. G. P. McCouch	"Patterns of Some Extra-ocular Reflexes in the Cat."
Dr. P. Bard	"The Behavior of a Cat without the Telencephalon."
Friday, August 1	
Dr. Paul S. Henshaw	"Some Biological Effects Produced by Alpha Particles on Drosophila Eggs."
Mr. Ware Cattell	"The Effect of X-Rays upon the Growth of the Wheat Seedling."
Dr. W. C. Curtis	"Effects of X-Rays upon Regeneration."
Dr. Charles Packard	"The Relation between Division Rate and Susceptibility to Radiation."
Friday, August 8	
Dr. Helen M. Miller	"Life Cycle of a Bisexual Rotifer."
Dr. Tracy M. Sonneborn	"Cause, Inheritance, and Effects of the Chain-forming Tendency in the Ciliate Protozoan, Colpidium."

Dr. Ruth Stocking Lynch	"The Effects of Long-continued Starvation in a Rotifer in Relation to Inheritance."
Dr. H. W. Stunkard	"The Life History of Cryptocotyle lingua."
Friday, August 15	
Dr. Mary S. MacDougall	"A Mutation in Chilodon uncinatus Produced by Ultra-violet Radiation —A Preliminary Report."
Dr. R. D. Manwell	"The Effect of Quinine and Plasmoquin on Avian Malaria."
Dr. E. Chatton	"The Asymmetric Motile Stages of Epistylis and the Question of the So-called Longitudinal Division of the Vorticellidae."
Dr. Ruth B. Howland	"Cine-photomicrograph of Microinjection of Vacuolated Protoplasm."
Friday, August 22	
Dr. Calvin B. Bridges	"The Neutralization of the Effects of Deficiencies through Duplications of the Same Chromosome Material."
Dr. A. H. Sturtevant	"A Peculiar Sex-ratio in Drosophila obscura."
Dr. Helen Redfield	"Studies of Crossing-over in Drosophila."
Dr. Jack Schultz	"The Eye of Pigments of Drosophila."
Friday, August 29	
Dr. H. B. Goodrich and Mr. I. B. Hansen	"Embryonic Development of Mendelian Characters in the Goldfish."
Dr. H. A. Abramson	"The Isoelectric Point of Mammalian Red Blood Cells."
Dr. E. N. Harvey and Mr. A. L. Loomis	"The Microscope-centrifuge."
Friday, September 5	
Dr. J. M. Johlin	"The Extraction of Micro-organisms."
Dr. W. E. Garrey and Dr. W. R. Bryan	"Alkalosis in Relation to Tetany following High Temperatures after Parathyroidectomy."
Dr. K. Blanchard	"Catalysis of Condensation Reactions by Amino-acids."
Dr. L. Michaelis and Dr. M. Schubert	"Metal Complex Compounds of Thioglycollic Acid,"

7. MEMBERS OF THE CORPORATION

1. Life Members

Allis, Mr. E. P., Jr., Palais Carnoles, Menton, France.
Andrews, Mrs. Gwendolen Foulke, Baltimore, Md.
Billings, Mr. R. C., 66 Franklin St., Boston, Mass.
Conklin, Prof. Edwin G., Princeton University, Princeton, N. J.
Coolidge, Mr. C. A., Ames Building, Boston, Mass.
Crane, Mr. C. R., New York City.
Evans, Mrs. Glendower, 12 Otis Place, Boston, Mass.
Fay, Miss S. B., 88 Mt. Vernon St., Boston, Mass.
Foot, Miss Katherine, Care of Morgan Harjes Cie, Paris, France.
Gardiner, Mrs. E. G., Woods Hole, Mass.
Jackson, Miss M. C., 88 Marlboro St., Boston, Mass.
Jackson, Mr. Chas. C., 24 Congress St., Boston, Mass.
Kidder, Mr. Nathaniel T., Milton, Mass.
King, Mr. Chas. A.
Lee, Mrs. Frederic S., 279 Madison Ave., New York City, N. Y.
Lowell, Mr. A. Lawrence, 17 Quincy St., Cambridge, Mass.
Means, Dr. James Howard, 15 Chestnut St., Boston, Mass.
Merriman, Mrs. Daniel, 73 Bay State Road, Boston, Mass.
Minns, Miss Susan, 14 Louisburg Square, Boston, Mass.
Morgan, Mr. J. Pierpont, Jr., Wall and Broad Sts., New York City, N. Y.
Morgan, Prof. T. H., Director of Biological Laboratory, California Institute of Technology, Pasadena, Calif.
Morgan, Mrs. T. H., Pasadena, Calif.
Noyes, Miss Eva J.
Osborn, Prof. Henry F., American Museum of Natural History, New York, N. Y.
Phillips, Mrs. John C., Windy Knob, Wenham, Mass.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pa.
Sears, Dr. Henry F., 86 Beacon St., Boston, Mass.
Shedd, Mr. E. A.
Thorndike, Dr. Edward L., Teachers College, Columbia University, New York City, N. Y.
Trelease, Prof. William, University of Illinois, Urbana, Ill.
Ware, Miss Mary L., 41 Brimmer St., Boston, Mass.
Williams, Mrs. Anna P., 505 Beacon St., Boston, Mass.
Wilson, Dr. E. B., Columbia University, New York City, N. Y.

2. Regular Members, August, 1930

Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Mass.

Addison, Dr. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pa.

Adolph, Dr. Edward F., University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

Allee, Dr. W. C., University of Chicago, Chicago, Ill.

Allen, Prof. Charles E., University of Wisconsin, Madison, Wis.

Allen, Prof. Ezra, New York Homeopathic Medical College, New York City, N. Y.

Allyn, Dr. Harriet M., Mount Holyoke College, South Hadley, Mass.

Amberson, Dr. William R., University of Tennessee, Memphis, Tenn.

Anderson, Dr. E. G., California Institute of Technology, Pasadena, Calif.

Austin, Dr. Mary L., Wellesley College, Wellesley, Mass.

Baitsell, Dr. George A., Yale University, New Haven, Conn.

Baker, Dr. E. H., 5312 Hyde Park Boulevard, Hyde Park Station, Chicago, Ill.

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THE RELATION BETWEEN CLEAVAGE AND TOTAL ACTIVATION IN ARTIFICIALLY ACTIVATED EGGS OF *URECHIS*

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It is generally assumed in most work on artificial parthenogenesis that cleavage and development result when the initial response of the egg to the artificial agent most closely resembles its response to the sperm. The percentage of eggs that respond in this fashion varies, of course, with the length of exposure to the artificial agent, presumably reaching a maximum for the exposure producing the highest percentage of activation.[1]

It would follow then that the cleavage-activation relation should be such that as the percentage of activation increases, the percentage of cleavage increases; that is, that the percentage of cleavage is directly proportional to the percentage of activation. Although this relation is practically always tacitly assumed in parthenogenesis experiments, detailed data on this point are lacking. If, however, exposures giving higher percentages of activation do not produce increasing percentages of eggs whose response is most nearly like that induced by the sperm, or if such eggs were not the ones which cleave and develop, an entirely different cleavage-activation relation might be expected. The determination of this relation is important, then, in an analysis of the factors which determine whether or not an artificially activated egg will cleave.

The variation of the percentage of activation with the length of exposure to the artificial agent is in itself a highly interesting fact, since it is not manifested in insemination of a normal batch of eggs with normal sperm. This variation may be attributed to variability in the amount of treatment necessary to activate a given egg, or, less likely perhaps, to a variation in the time at which the change produced by the activating agent reaches a given egg. Whatever its source, the way in which the percentage of activation varies with the length of exposure is useful in helping to elucidate the mechanism by which the artificial agent activates the egg.

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[1] Any egg in which initial developmental changes have taken place will be termed "activated" in this paper, regardless of maturation or cleavage.

In the parthenogenesis experiments on *Urechis* eggs a unique relation between cleavage and activation was found, such that as the percentage of activation increases, the percentage of cleavage decreases. The variation of percentage activation with length of exposure was found to give a particular type of distribution curve in certain of the experiments. These results together with their interpretation are presented in detail in this report.

MATERIAL AND METHOD

The eggs used in these experiments were those of the echiuroid, *Urechis caupo*, described by Fisher and MacGinitie (1928). The changes undergone by the egg upon normal fertilization, and upon artificial activation, and the method used in activating the eggs were described in detail in a previous publication (Tyler, 1931). Briefly, it was found that dilutions of sea water ranging from 80 per cent to distilled water were effective in activating the *Urechis* eggs.[2] In order to treat the eggs, a batch was transferred with as little sea water as possible to a Stender dish containing a large volume of the hypotonic solution. Samples were then removed after various intervals of time to Syracuse dishes containing normal sea water. All the usual precautions in regard to contamination by sperm or foreign matter, hypertonicity, etc., were taken.

TABLE I

Unfertilized Eggs Treated with Distilled Water, Temperature 21.8° C.

Length of Exposure	Activation	Cleavage of Activated Eggs
min.	*per cent*	*per cent*
0.05	. 57.0	32.4
0.08	95.0	10.0
0.17	99.6	0.6
0.25	100.0	0.0
0.33	100.0	0.05
0.42	100.0	0.1
0.50	100.0	0.0
0.67	100.0	0.0
0.83	100.0	0.0
1.00	100.0	1.5
1.50	100.0	0.0
2.00	100.0	0.0
3.00	100.0	0.0
4.00	100.0	0.0
5.00	99.0	0.0

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[2] Eighty per cent sea water, for example, is made up of eight parts sea water and two parts distilled water. The sea water used was always taken at the same height of tide.

The percentages of cleavage and of activation were based on counts of at least three hundred eggs; frequently, especially for very low or very high percentages of activation, a much larger number were counted.

It was shown that two types of activated eggs appear as a result of the treatment. One type is characterized by initial changes which are indistinguishable from those induced by the sperm. In this type the breakdown of the germinal vesicle, the rounding out of the indentation, the elevation of the membrane, and the extrusion of polar bodies occur in very much the same manner as when the egg is fertilized by a sperm. The time relations for these various changes, allowing for the time of exposure, compare very closely with the time schedule of the same events in the fertilized egg. However, in spite of the remarkable similarity in behaviour of this type of artificially activated egg to that of the fertilized egg, none of the eggs divide.[3]

The other type of artificially activated egg departs widely in its behaviour from that of the normal fertilized egg. The only visible

TABLE II

Unfertilized Eggs Treated with 20 Per Cent Sea Water, Temperature 22.0° C.

Length of Exposure	Activation	Cleavage of Activated Eggs
min.	*per cent*	*per cent*
0.08	25.0	69.2
0.17	58.1	58.7
0.25	92.0	31.4
0.33	98.8	17.3
0.50	100.0	3.1
0.67	100.0	1.0
0.83	100.0	4.2
1.00	100.0	0.5
1.17	100.0	0.1
1.33	99.5	2.2
1.50	100.0	0.1
1.67	100.0	0.0
1.83	100.0	0.0
2.00	100.0	0.0
2.50	100.0	0.0
3.00	100.0	0.0
3.50	100.0	0.0
4.00	100.0	0.0
5.00	100.0	0.0
7.00	100.0	0.1
10.00	100.0	0.3
15.00	100.0	0.6
20.00	100.0	1.5
40.00	100.0	0.0

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[3] In only three cases were eggs with two polar bodies seen to divide. The three eggs proceeded only as far as the two-cell stage.

change that occurs in this type of egg within the first three-quarters of an hour after treatment is the dissolution of the germinal vesicle. The egg remains indented, no membrane elevation occurs, and no polar bodies are extruded. After that time the eggs begin to round up, and lift off membranes, but no polar bodies appear. Practically all of the eggs of this type divide, the time of first division varying from one hour and twenty minutes to about three hours. The eggs which cleave and develop are thus the ones which show a poor initial response to the treatment. In what follows, then, the percentage of cleavage is practically identical with the percentage of "poorly activated eggs," and the data on percentage of cleavage and of activation will also show the relation between the percentage of "imperfectly" and of "perfectly" activated eggs for various strengths of hypotonic solutions.

The Variation of Percentage of Activation and of Cleavage with Length of Exposure for Various Dilutions of Sea Water

Treatment with Distilled Water

The action of distilled water is extremely rapid in causing activation of the eggs. After 3 seconds' treatment, 57 per cent of the eggs

Table III

Unfertilized Eggs Treated with 30 Per Cent Sea Water, Temperature 22.1° C.

Length of Exposure	Activation	Cleavage of Activated Eggs
min	*per cent*	*per cent*
0.17	3.8	37.5
0.33	59.8	13.8
0.50	99.7	1.4
0.67	99.6	0.9
0.83	100.0	0.4
1.00	100.0	0.6
1.33	100.0	0.4
1.67	98.1	3.1
2.00	99.2	0.7
2.50	99.0	0.5
3.00	99.3	0.8
3.50	98.5	1.6
4.00	83.6	2.3
4.50	86.1	3.2
5.00	81.8	11.1
6.00	86.7	1.0
8.00	100.0	0.0
10.00	100.0	0.1
15.00	100.0	0.0
20.00	100.0	0.0
40.00	100.0	0.0

become activated, and after 15 seconds all of the eggs are activated. The results of one series of exposures are given in Table I. Another series run in the same manner gave quite similar results. The percentage of the activated eggs that cleave (column three in the table) is seen to drop very rapidly as the percentage of activation increases. Thus, when 100 per cent activation is obtained, there is practically no cleavage.

Cytolysis sets in after 2 minutes' exposure and reaches 90 per cent at 5 minutes' treatment. The activated eggs in that range are somewhat abnormal in appearance, having a relatively wide membrane and forming blisters over the surface so that polar bodies are often indistinguishable.

Treatment with Twenty Per Cent Sea Water

The action of 20 per cent sea water is less rapid than that of distilled water. The results of one series are given in Table II.

TABLE IV

Unfertilized Eggs Treated with 40 Per Cent Sea Water, Temperature 22.0° C.

Length of Exposure	Activation	Cleavage of Activated Eggs
min.	*per cent*	*per cent*
0.17	0.0	0.0
0.33	52.1	31.4
0.50	94.0	13.5
0.67	97.8	2.3
0.83	99.7	0.9
1.00	99.9	0.1
1.33	100.0	0.1
1.67	100.0	0.2
2.00	99.6	0.0
2.50	99.0	2.4
3.00	98.8	1.1
3.50	98.3	1.6
4.00	97.5	3.2
5.00	85.3	6.4
7.00	88.0	0.9
10.00	99.9	0.4
15.00	100.0	0.0
20.00	100.0	0.0
40.00	100.0	0.0

Fifteen seconds longer treatment is required to give 100 per cent activation than for the distilled water. The rise in percentage of activation with time of exposure is again seen to be accompanied by a drop in cleavage. No exceptions are seen in the first part of the table and the ones occurring in the latter part are of small magnitude. Cytolysis sets in after 4 minutes' exposure and reaches 70 per cent after 15 minutes and 90 per cent after 20 minutes. Increasing numbers of abnormal eggs of the type described above are found in that range. Two other series of experiments were run, and closely similar results obtained.

TABLE V

Unfertilized Eggs Treated with 45 Per Cent Sea Water, Temperature 21.0° C.

Length of Exposure	Activation	Cleavage of Activated Eggs	Volume in $\mu^3 \times 10^5$
min.	*per cent*	*per cent*	
0.17	0.2	0.0	
0.33	11.5	50.0	
0.50	39.0	29.2	
0.67	81.3	10.6	
0.83	92.9	4.8	
1.00	98.6	1.4	8.12
1.33	100.0	0.2	
1.67	100.0	0.0	
2.00	99.5	0.0	8.90
2.50	98.3	0.3	
3.00	96.4	0.8	9.51
3.50	87.3	2.2	
4.00	65.7	4.0	10.10
5.00	34.4	20.5	10.45
6.00	21.2	26.2	10.92
8.00	7.9	22.2	11.49
10.00	6.9	50.0	11.92
15.00	0.8	43.0	12.95
20.00	1.3	51.6	13.51
40.00	3.3	8.3	13.96

Treatment with Thirty Per Cent Sea Water

With 30 per cent sea water the percentage of activation rises less rapidly than with 20 per cent. The results again show that as the percentage of activation increases, the percentage of cleavage decreases. Table III gives the results of one series. The percentage of activation shows a slight drop after about one and one-half minutes' exposure which becomes quite marked at 4 to 6 minutes' exposure. But as the activation drops, the cleavage is seen to rise, so that at 5 minutes' exposure, where the activation has dropped to 82 per cent, the cleavage has risen to 11 per cent. The activation then rises again to 100 per cent and the cleavage drops to zero.

Cytolysis sets in after 6 minutes' treatment and reaches 50 per cent after 40 minutes. The abnormal eggs referred to above again appear in this range of exposures.

Four other series of experiments were run, at temperatures ranging from sixteen to twenty-three degrees, and very similar results obtained.

The inverse relation between cleavage and activation was evident in each series. If every case in which an increase (or decrease) in activation accompanied by an increase (or decrease) in cleavage to the extent of at least one per cent is considered an exception, then out of a total of eighty-one dishes there are seven exceptions.

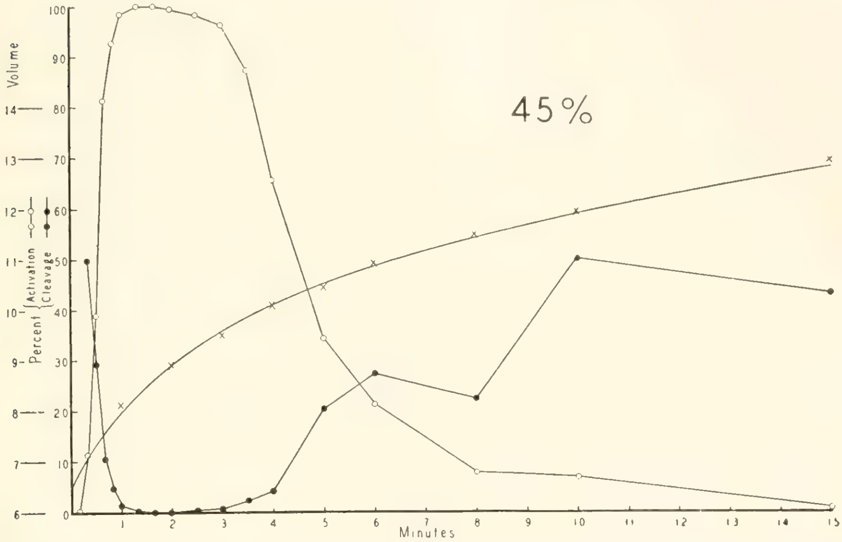

FIG. 1. Variation of percentage activation (open circles), percentage cleavage (solid circles) and mean volume of eggs (continuous curve) with length of exposure to 45 per cent sea water. Data of Table V.

Treatment with Forty Per Cent Sea Water

The percentage of activation for eggs treated with 40 per cent sea water rises less rapidly than for eggs treated with any of the preceding dilutions. Table IV gives the results of one series of exposures. The activation is seen to rise rapidly to 100 per cent, drop more slowly to 85 per cent and return again to 100 per cent. The percentage of cleavage of the activated eggs decreases as the activation increases, and increases as the activation drops. The inverse relation between cleavage and activation is thus again clearly shown, only one exception occurring in the table, namely at the three minute exposure, where a drop in activation is followed by a drop in cleavage greater than one per cent.

At the 8 minutes' exposure there is 4 per cent of cytolysis, which increases to about 30 per cent for the 40 minutes' treatment. The abnormal eggs again occur in this range.

Three other series were run with 40 per cent sea water, totaling forty-seven dishes. Out of these a total of four exceptions of magnitude greater than one per cent were obtained.

TABLE VI

Unfertilized Eggs Treated with 50 Per Cent Sea Water, Temperature 21.5° C.

Length of Exposure	Activation	Cleavage of Activated Eggs	Volume in $\mu^3 \times 10^{-5}$
min.	*per cent*	*per cent*	
0.17	0.1	100.0	
0.33	2.0	80.1	
0.50	43.2	40.6	
0.67	91.0	21.6	
0.83	96.7	10.6	
1.00	99.2	5.3	7.83
1.33	99.8	0.7	
1.67	100.0	0.4	
2.00	100.0	0.4	8.72
2.50	100.0	1.2	
3.00	90.8	26.9	9.29
3.50	93.4	21.9	
4.00	86.0	39.2	9.72
4.50	68.3	63.2	
5.00	33.9	76.2	10.10
6.00	34.9	80.0	10.46
8.00	18.8	94.7	11.08
10.00	7.1	83.3	11.42
15.00	0.5	40.0	12.20
20.00	0.4	79.0	12.72
40.00	1.5	61.3	12.85

Treatment with Forty-five Per Cent Sea Water

The results obtained with 45 per cent sea water differ in two respects from those obtained with the preceding dilutions of sea water. These are, first, that the percentage of activation returns practically to zero after its initial rise to 100 per cent, and second, that very little cytolysis sets in.

In Table V the results of one series of experiments are presented. The rate of increase in activation is slower than with the preceding dilutions. After 8 minutes' exposure a few of the eggs become cytolysed and the amount of cytolysis reaches 5 per cent after 40 minutes.

The inverse relation between cleavage and activation is quite evident in the table and is illustrated graphically in Fig. 1. The "exceptions" generally occur in the dishes showing low percentages of activation. Similar results were obtained in three other series of experiments run with 45 per cent sea water. Out of a total of seventy-three dishes examined, sixteen exceptions were found, all of them in dishes showing less than 8 per cent activation.

The increase in activation occurs much more rapidly than the decrease. This may readily be seen in the graph (Fig. 1), where the percentage of activation plotted against time gives a skew curve. The probable interpretation of this result will be presented later. But in connection with the activation-time curve, it is of interest to present here the curve showing the increase in volume with length of exposure to the 45 per cent sea water. The data from which the curve was drawn are given in Table V. Each point represents the average of the volumes of three eggs. The measurements of the diameters were made with a Filar ocular micrometer. With this micrometer measurements accurate to 0.1 per cent may be obtained. However, the variations in volume, for the data presented here and below, ranged as high as 5 per cent. This is probably due to the rapid change in volume that is taking place as the measurements are made and to the variability of the eggs. The volume measurements are being repeated on a larger scale and by means of a cinematograph in order to obtain accurate data for an analysis of the swelling process itself. But even the relatively rough data presented here will be shown to be useful in an analysis of the activation-time curves obtained in these experiments.

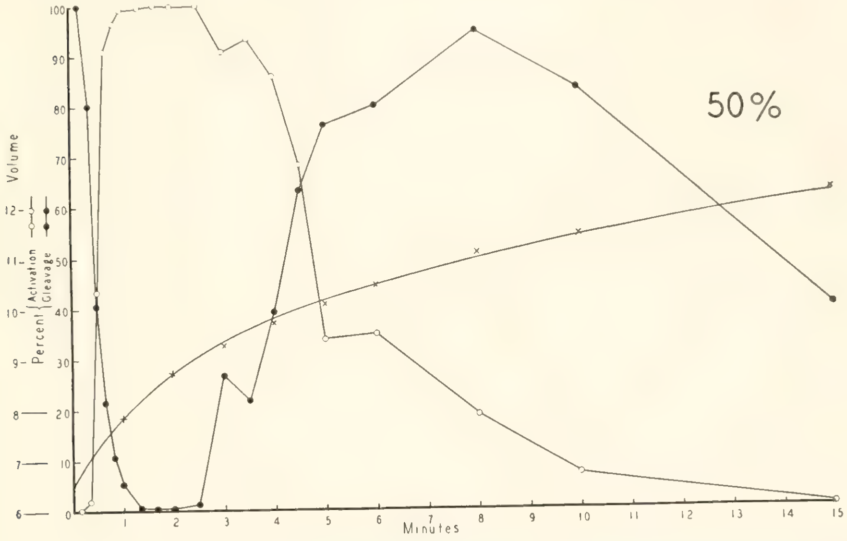

FIG. 2. Variation of percentage activation (open circles), percentage cleavage (solid circles) and mean volume of eggs (continuous curve) with length of exposure to 50 per cent sea water. Data of Table VI.

The swelling curve of Fig. 1 shows that the eggs continue to increase in volume even after the percentage of activation begins to drop. The curve itself is of the exponential type, the slope continually decreasing. In other words, the increase in volume occurs less rapidly as the time of exposure increases.

Table VII

Unfertilized Eggs Treated with 55 Per Cent Sea Water, Temperature 21.2° C.

Length of Exposure	Activation	Cleavage of Activated Eggs	Volume in $\mu^3 \times 10^{-5}$
min.	*per cent*	*per cent*	
0.17	0.4	100.0	
0.33	0.9	100.0	
0.50	10.0	53.3	
0.67	38.4	37.4	
0.83	70.3	29.4	
1.00	89.5	17.6	7.74
1.33	96.2	9.6	
1.67	98.4	2.0	
2.00	99.9	1.0	8.73
2.50	97.8	1.6	
3.00	93.3	5.6	9.32
3.50	88.8	7.1	
4.00	85.6	12.4	9.57
5.00	66.4	21.3	9.82
6.00	64.1	21.0	10.11
8.00	47.3	16.6	10.49
10.00	13.9	15.4	10.89
12.00	1.7	44.2	
15.00	1.1	70.6	11.41
20.00	0.0	0.0	11.63
40.00	2.2	90.9	11.84

Treatment with Fifty Per Cent Sea Water

The results obtained with 50 per cent sea water are quite similar to those obtained with 45 per cent sea water, except that the increase in activation occurs more slowly and practically no cytolysis occurs in any of the dishes. The increase in volume of the eggs in 50 per cent sea water is also somewhat slower than for those exposed to 45 per cent sea water, and the equilibrium volume attained is, of course, smaller.

Table VI contains the results of one series of experiments and a set of volume measurements (means of three eggs) obtained at different times. The data are presented graphically in Fig. 2. The results again bear out the inverse relation between cleavage and activation. It is interesting to note that the irregular rise in the activation curve at the three and one-half minutes' exposure is accompanied by a drop in cleavage. The exceptions occur only in the last four points of the graph, where the percentage of activation is low. A total of eighty-two dishes in five series of experiments gave sixteen exceptions all of the same type illustrated here.

The activation shows a drop to practically zero after its initial rise

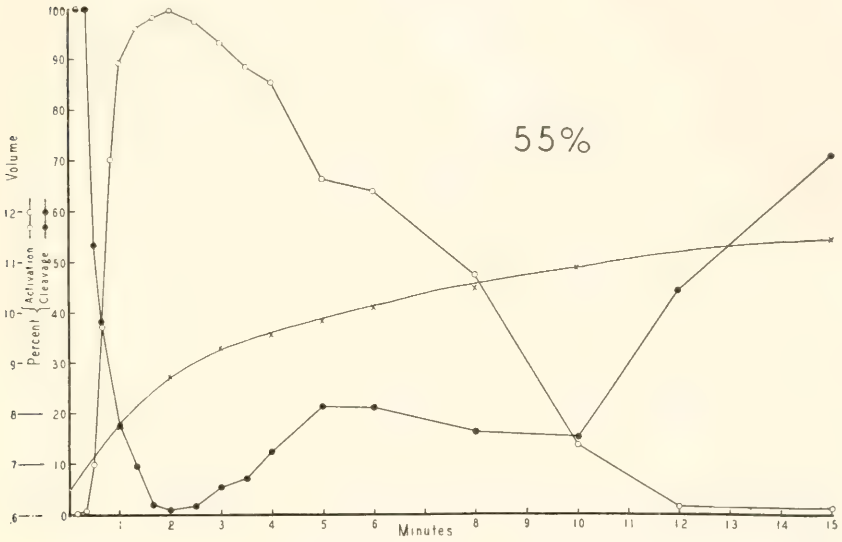

FIG. 3. Variation of percentage activation (open circles), percentage cleavage (solid circles) and mean volume of eggs (continuous curve) with length of exposure to 55 per cent sea water. Data of Table VII.

to 100 per cent, as in the preceding case. The rise in activation is again seen to occur more rapidly than the subsequent decrease, giving the skew activation-time curve shown in Fig. 2. The volume curve for the eggs in the 50 per cent sea water is of the same type as obtained with the preceding dilution.

Treatment with Fifty-five Per Cent Sea Water

The results of one series of experiments with 55 per cent sea water and the volume data for the same dilution are presented in Table VII and Fig. 3. The data again show a decrease in cleavage as the activation increases and an increase in cleavage as the activation decreases. Four series of experiments totaling seventy-four dishes gave twelve exceptions—chiefly at low percentages of activation.

The activation-time curve is of the same shape as that obtained in the preceding case, but it is shifted slightly to the right, so that the time required for the maximum percentage of activation and for the return to zero per cent activation is longer than with 50 per cent sea water.

The swelling curve (Fig. 3) shows that the volume continues to increase after the percentage of activation has reached a maximum. It is also of the exponential type in which the rate of increase in volume decreases with time.

Treatment with Sixty Per Cent Sea Water

The results obtained with 60 per cent sea water again differ from the preceding only in the time relations of activation and cleavage and the volume curve. The data is given in Table VIII, and graphically represented in Fig. 4.

TABLE VIII

Unfertilized Eggs Treated with 60 Per Cent Sea Water, Temperature 22.3° C.

Length of Exposure	Activation	Cleavage of Activated Eggs	Volume in $\mu^3 \times 10^{-5}$
min.	*per cent*	*per cent*	
0.17	0.1	0.0	
0.33	1.0	0.0	
0.50	1.0	50.0	
0.67	13.4	33.3	
0.83	30.3	25.8	
1.00	61.3	25.9	7.88
1.33	73.1	13.7	
1.67	88.7	7.7	
2.00	99.4	0.4	8.68
2.50	100.0	0.1	
3.00	100.0	0.2	9.16
3.50	97.6	2.9	
4.00	88.2	9.3	9.45
5.00	85.7	5.5	9.69
6.00	75.0	30.6	10.01
7.00	42.4	46.5	
8.00	16.0	61.5	10.16
10.00	14.7	81.2	10.49
12.00	9.5	37.1	
15.00	5.1	72.3	10.94
20.00	0.0	0.0	11.10
40.00	0.0	0.0	11.11

The cleavage-activation relation shows up quite clearly. Out of eighty-nine dishes in five series of experiments, fourteen relatively unimportant exceptions were obtained.

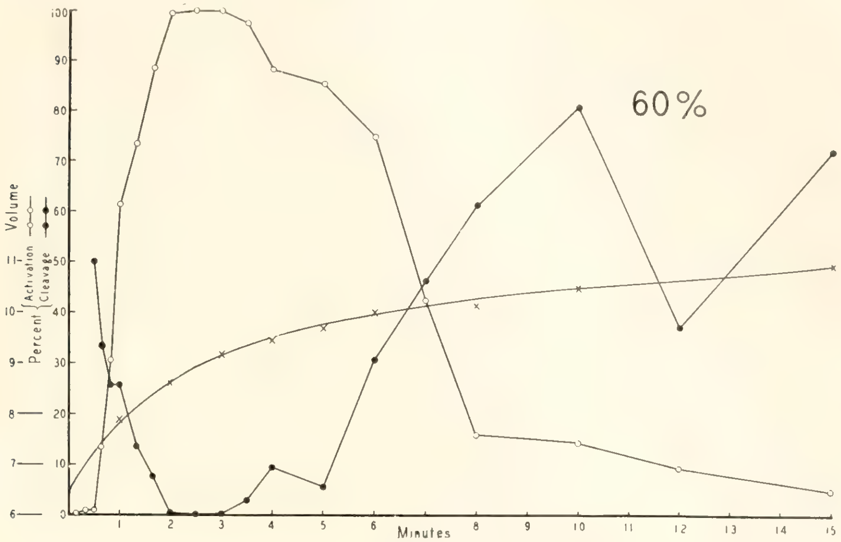

FIG. 4. Variation of percentage activation (open circles), percentage cleavage (solid circles) and mean volume of eggs (continuous curve) with length of exposure to 60 per cent sea water. Data of Table VIII.

The activation-time curve shows a slight shift to the right when compared with the preceding ones, but its asymmetry is still quite evident.

The swelling curve is of the same type as in the preceding cases but approaches a lower equilibrium volume.

Treatment with Sixty-five Per Cent Sea Water

Sixty-five per cent sea water gives results which differ from the preceding in the same direction as the results obtained with the 60 per cent sea water differ from those obtained with 55 per cent. One series of experiments and a set of mean volumes are shown in Table IX and Fig. 5.

The inverse relation between cleavage and activation is evident in spite of certain relatively large irregularities. From three series of experiments seven relatively large and eight minor exceptions were obtained out of a total of fifty-nine dishes.

TABLE IX

Unfertilized Eggs Treated with 65 Per Cent Sea Water, Temperature 22.0° C.

Length of Exposure	Activation	Cleavage of Activated Eggs	Volume in $\mu^3 \times 10^{-5}$
min.	*per cent*	*per cent*	
0.17	0.0	0.0	
0.33	0.2	0.0	
0.50	0.0	0.0	
0.67	0.7	40.0	
0.83	6.4	36.3	
1.00	13.6	11.8	7.61
1.33	77.8	20.8	
1.67	87.6	13.4	
2.00	92.0	10.2	8.32
2.50	96.5	6.1	
3.00	98.6	2.1	8.73
3.50	92.5	6.7	
4.00	71.9	22.5	9.25
5.00	49.0	56.7	9.56
6.00	21.8	46.9	9.49
7.00	20.3	51.0	
8.00	13.8	42.4	9.88
10.00	13.7	34.9	10.15
12.00	6.0	40.9	
15.00	3.9	15.3	10.50
20.00	0.2	100.0	10.55
40.00	0.0	0.0	10.55

The activation-time curve (Fig. 5) is asymmetrical as in the preceding cases, but it shows a slight shift to the right.

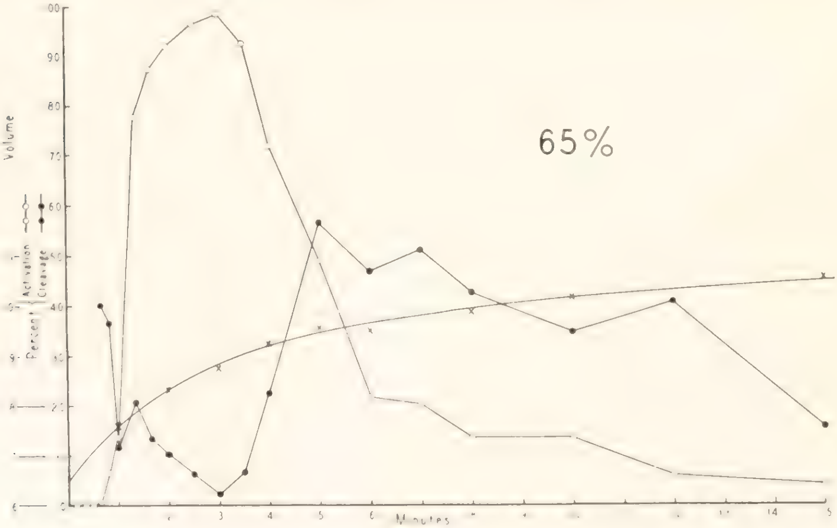

FIG. 5. Variation of percentage activation (open circles), percentage cleavage solid circles) and mean volume of eggs (continuous curve) with length of exposure to 65 per cent sea water. Data of Table IX.

The volume increase is slower than for the eggs in more dilute sea water, and approaches a lower asymptotic value.

Treatment with Seventy Per Cent Sea Water

The results of one series run with 70 per cent sea water are tabulated in Table X. Figure 6 shows that trend of the data graphically.

TABLE X

Unfertilized Eggs Treated with 70 Per Cent Sea Water, Temperature 21.2° C.

Length of Exposure	Activation	Cleavage of Activated Eggs
min.	*per cent*	*per cent*
0.17	0.4	57.1
0.33	0.8	50.0
0.50	2.5	55.2
0.67	2.4	29.8
0.83	4.1	30.2
1.00	4.6	29.5
1.33	6.4	20.3
1.67	20.4	19.5
2.00	46.1	10.2
2.50	96.2	9.1
3.00	100.0	2.3
3.50	97.5	10.9
4.00	69.2	19.7
4.50	51.6	35.7
5.00	31.6	55.5
6.00	16.7	65.2
8.00	7.4	82.9
10.00	13.3	76.3
15.00	12.7	85.8
20.00	4.6	83.3
40.00	0.3	66.7

No serious divergence from the cleavage-activation relation is evident. Two series of experiments totaling thirty-seven dishes gave seven minor variations.

The activation-time curve (Fig. 6) is again decidedly asymmetrical. It is displaced to the right, so that the return to zero per cent activation requires a longer exposure than in the preceding cases.

The volume data are not presented for this or for the succeeding dilutions of sea water. The volume increase proceeds more slowly, of course, and reaches a smaller equilibrium volume with increasing concentrations of sea water.

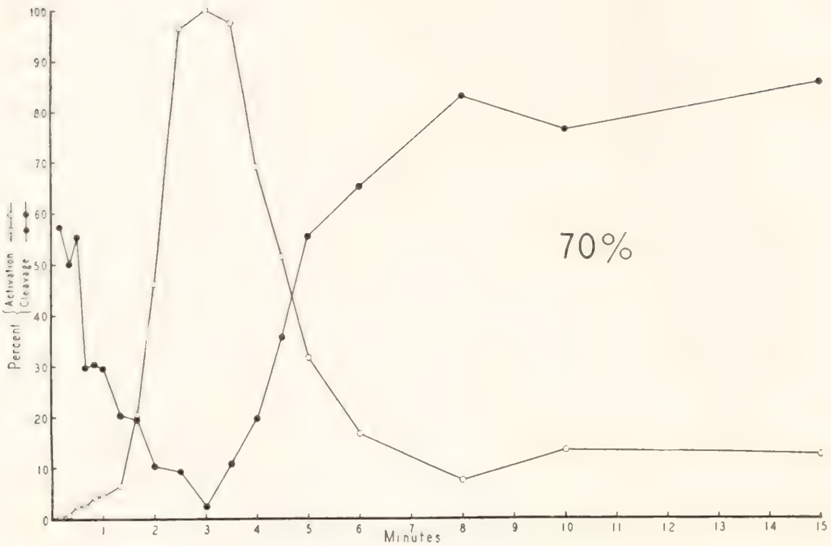

FIG. 6. Variation of percentage activation (open circles) and percentage cleavage (solid circles) with time of exposure to 70 per cent sea water. Data of Table X.

Treatment with Seventy-five Per Cent Sea Water

Table XI and Fig. 7 contain the results of one series of experiments with 75 per cent sea water.

TABLE XI

Unfertilized Eggs Treated with 75 Per Cent Sea Water, Temperature 20.8° C.

Length of Exposure	Activation	Cleavage of Activated Eggs
min.	*per cent*	*per cent*
0.17	0.0	0.0
0.33	0.0	0.0
0.50	0.0	0.0
0.67	0.0	0.0
0.83	0.0	0.0
1.00	0.0	0.0
1.33	3.3	71.4
1.67	8.2	50.0
2.00	33.9	45.9
2.50	83.2	49.5
3.00	86.2	32.3
3.50	90.0	28.4
4.00	79.3	28.6
5.00	47.8	59.2
6.00	30.2	65.0
8.00	7.4	61.3
10.00	1.2	66.7
15.00	0.1	40.0
20.00	0.0	0.0

As before, the percentage of cleavage varies inversely with the percentage of activation, although the difference between the maximum of activation and the corresponding minimum of cleavage is not as great as in the cases listed above. Two series of experiments totaling thirty-one dishes gave four exceptions.

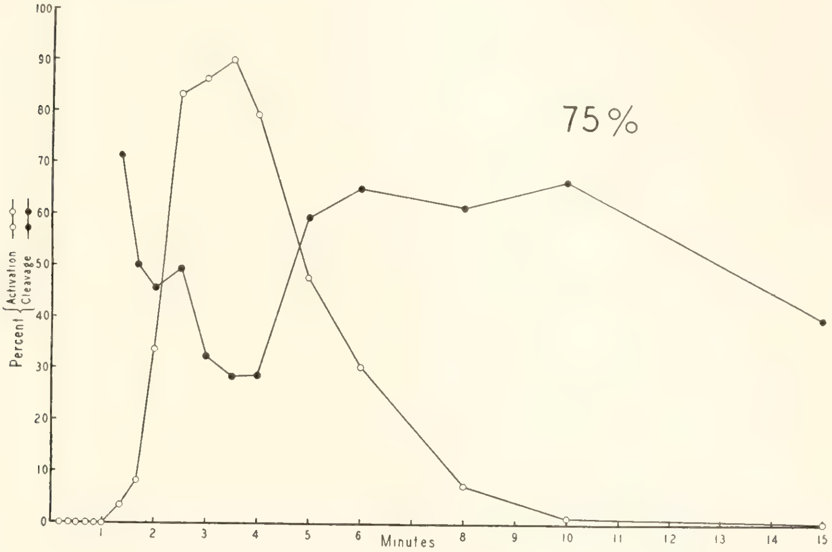

FIG. 7. Variation of percentage activation (open circles) and percentage cleavage (solid circles) with time of exposure to 75 per cent sea water. Data of Table XI.

For the activation-time curve (Fig. 7), the time to reach a maximum is longer than in the preceding case, but the drop to zero per cent occurs sooner. However, the maximum value reached is only 90 per cent activation as compared with 100 per cent in the previous cases. The curve itself is still asymmetrical.

Treatment with Eighty Per Cent Sea Water

Eighty per cent sea water generally fails to give more than one to two per cent activation. In one series of experiments, however, an exceptionally high percentage of activation was obtained. The results are given in Table XII and Fig. 8.

It is readily seen from the data that practically every increase (or decrease) in activation is accompanied by a decrease (or increase) in cleavage, bearing out the inverse relation between cleavage and activation.

The activation-time curve reaches its maximum as quickly as for the 75 per cent sea water, but that is undoubtedly due to the higher temperature at which this series was run. The activation curve does not return to zero, but maintains a relatively high percentage of activation and a correspondingly high percentage of cleavage.

TABLE XII

Unfertilized Eggs Treated with 80 Per Cent Sea Water, Temperature 22.5° F.

Length of Exposure	Activation	Cleavage of Activated Eggs
min.	*per cent*	*per cent*
0.17	0.0	0.0
0.33	0.0	0.0
0.50	0.0	0.0
0.67	0.0	0.0
0.83	0.2	100.0
1.00	1.4	42.8
1.33	6.6	31.2
1.67	14.5	30.8
2.00	58.2	26.8
2.50	94.1	21.5
3.00	95.7	17.6
3.50	98.8	3.5
4.00	98.7	8.3
5.00	81.6	57.8
6.00	92.5	37.1
8.00	80.7	48.6
10.00	65.5	59.1
15.00	64.1	38.2
20.00	62.5	48.8
40.00	47.6	64.2

THE VARIATION OF PERCENTAGE OF ACTIVATION WITH VOLUME FOR VARIOUS DILUTIONS OF SEA WATER

When the percentage of activation is plotted against the mean volume attained by the eggs at different lengths of exposure, a curve is obtained which is much more symmetrical than the activation-time curve. Figure 9 shows five curves of that type, for 45, 50, 55, 60 and 65 per cent sea water. The percentages of activation were plotted in each case against the volumes attained at corresponding times of exposures, the volumes being taken from the smooth curves.

The activation-volume curves of Fig. 9 approach in shape the normal distribution curve. The individual curves have the same abscissa but the ordinates are raised successively for each dilution of sea water. It can readily be seen that even with the same coördinates the curves would not coincide; but their divergence is no greater than would be expected when one considers the statistical nature of the activation values and the errors involved in the volume measurements. Moreover, there are probably injury factors operative in the lower concentrations of sea water that are not present in the higher concentrations, as indicated by the cytolysis obtained in 45 per cent sea water.

Fertilization of "Over-Exposed" Eggs

The activation-time curves for concentrations of sea water above 40 per cent are seen to rise to a maximum of about 100 per cent activation and then drop off to zero.

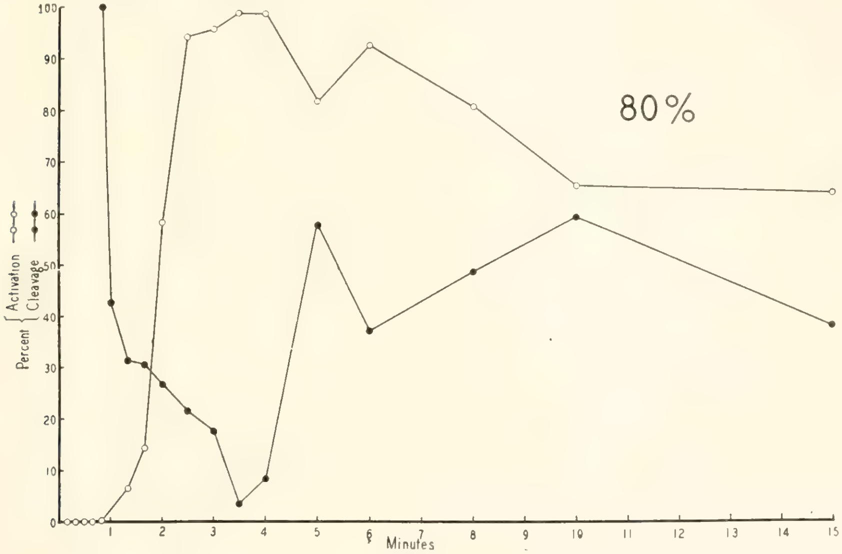

Fig. 8. Variation of percentage activation (open circles) and percentage cleavage (solid circles) with time of exposure to 70 per cent sea water. Data of Table XII.

The eggs which do not respond before the "optimum exposure" is reached may be termed "under-exposed" unactivated eggs, and those which do not respond upon longer exposures may be termed "over-exposed" unactivated eggs.

The failure of the "over-exposed" unactivated eggs to respond to the treatment might presumably be due to an injury effect, or other change produced in the eggs. The "over-exposed" unactivated eggs as well as the under-exposed unactivated eggs were therefore inseminated with fresh sperm in order to determine whether they would become fertilized and produce normal embryos. The results obtained with 45 to 65 per cent sea water are given in Table XIII. The third column in the table gives the total percentages of activation obtained with the lengths of exposure listed in column two. The fourth column gives the percentage of the unactivated eggs that become fertilized upon addition of sperm, and the fifth column, the percentage of the fertilized eggs that produce normal larvæ.

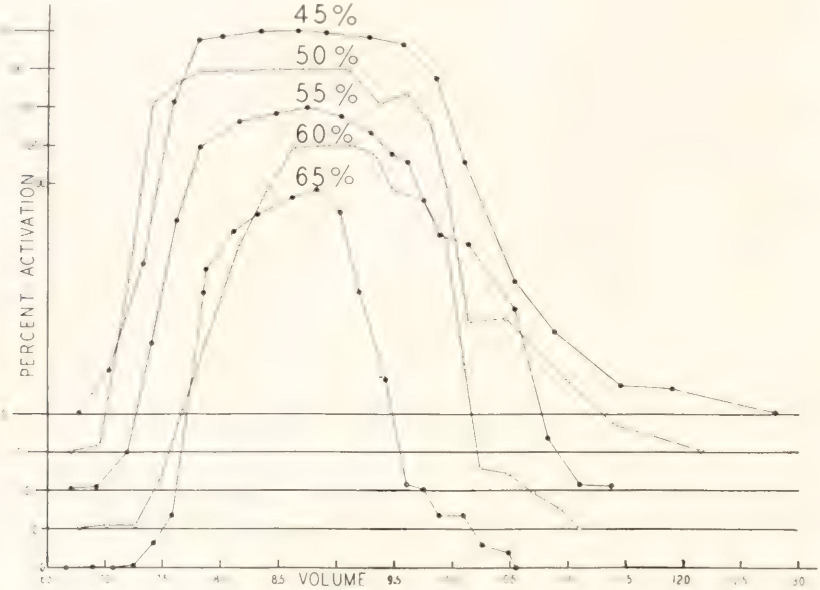

FIG. 9. Variation of percentage of activation with mean volume of eggs attained at corresponding times of exposure to 65, 60, 55, 50, and 45 per cent sea water. Ordinates raised successively for each dilution of sea water. Data from Tables V to IX; volumes taken from the smooth curves of Figs. 1 to 5.

The unactivated eggs were transferred to a separate dish and inseminated at about 2 to 3 hours after treatment. Control eggs (listed in the table as 0.0 minutes' exposure) were inseminated at the same time.

The "under-exposed" unactivated eggs are not given for the 55 and the 60 per cent sea water. In the other three cases the "under-exposed" unactivated eggs show practically 100 per cent fertilization and a high percentage of normal embryos. The "over-exposed" eggs show a high percentage of fertilization in every case, comparing quite favorably with that given by the control eggs. The percentage of normal embryos obtained varies considerably, but is quite as good as that obtained from the controls, except for the 45 per cent sea water. However, in the latter case a relatively large percentage of the eggs were polyspermic.

Table XIII

Insemination of "Under-Exposed" and "Over-Exposed" Unactivated Eggs

Concentration of Sea Water	Length of Exposure	Activation	Fertilization	Normal Embryos
per cent	*min.*	*per cent*	*per cent*	*per cent*
45	0.0	—	95	40
	0.17	0.0	99	50
	1.50	100.0	—	—
	10.00	5.7	70	20
	15.00	0.0	60	2
	20.00	0.3	60	5
	40.00	0.5	60	5
50	0.0	—	98	25
	0.17	0.1	100	60
	2.00	100.0	—	—
	15.00	0.5	80	70
55	0.0	—	65	70
	2.00	100.0	—	—
	20.00	0.0	50	65
60	0.0	—	100	100
	2.75	100.0	—	—
	7.00	42.4	100	100
	10.00	14.7	100	95
	20.00	0.0	100	100
65	0.0	—	100	65
	0.17	0.0	100	100
	3.00	99.0	—	—
	7.00	28.1	100	40
	10.00	18.3	90	75
	20.00	0.0	99	50

The results show that the "over-exposed" unactivated eggs are still capable of becoming fertilized, even though a shorter exposure would have resulted in every egg becoming activated upon return to normal sea water.

Discussion

1. *Variation of Rate of Increase in Activation with Dilution of Sea-Water*

It is evident from the results presented above that the factors causing activation are brought into action more quickly, the lower the concentration of the sea water used for the treatment. In the dilute sea water the egg swells due to intake of water. The volume increase also occurs more quickly, the lower the concentration of the sea water in which the eggs are allowed to swell. This parallel behaviour suggests that volume increase in the dilute sea water may be used as a basis for an interpretation of the results presented above; but this is not meant to imply that water-intake alone is responsible for the activation of the egg.[4]

2. *Activation-Time Curves*

For concentrations of sea water ranging from 45 to 75 per cent the percentage of activation was seen to rise rapidly to a maximum and then fall off more slowly. In terms of volume change this means that when the egg is in a definite volume range it will become activated upon return to normal sea water, but before or after passing through that volume range the egg does not become activated upon return to normal sea water. This volume range is evidently well below the equilibrium volume, since the eggs continue to swell after the time of exposure giving the maximum activation. The reason why a range of volumes rather than one definite volume is specified will be indicated below. There is considerable variability in the time at which different eggs pass through the same volume range when a given batch is exposed to a given dilution of sea water. Thus some of the eggs will have reached the volume range from which return to normal sea water results in activation before the others have entered that range. Correspondingly, some of the eggs will have passed through that volume range while the others are still in it. Let us term the volume range resulting in activation the "optimum volume range." The percentage of eggs passing through a given volume range at a given time will depend on the kind of variability shown by the eggs. If the variability of this material is expressed by the normal distribution curve, then we would expect the variability in the percentage of eggs passing through the "optimum volume range" to be expressed by that type of curve only if the increase in volume were a linear function of the time of exposure. But the volume increase is a logarithmic function of time, the rate of swelling continually decreasing with time of exposure. The eggs therefore enter the "optimum volume range" more rapidly than they leave it. Thus the variation in the percentage of eggs passing through the "optimum volume range" with time of exposure should be expressed by a skew distribution curve with its mode displaced to the left. In other words, the variation of percentage activation with time of exposure should give a skew curve, since the percentage of eggs passing through the "optimum volume range" is by definition identical with the percentage of activation. This is in fact the type of curve that is obtained when percentage of activation is plotted against length of exposure (Figs. 1 to 8).

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[4] The change in hydrogen ion concentration, for example, might be an important factor. It ranged from pH 8.2 for the sea water to pH 7.1 for the distilled water used.

The reason for assuming a range of volumes rather than one definite optimum volume results from the following consideration. The maximum of the activation-time curve is at 100 per cent activation. This means that all of the eggs must be in such a condition after a certain time of exposure that removal to normal sea water at that time results in every egg becoming activated. But the volume measurements show that the eggs vary in the time of exposure at which a given volume is reached. Therefore, if we adhere to the volume interpretation we must assume that a range of volumes, at least as great as the variation in volume of the individual eggs, is effective in causing activation upon return to ordinary sea water. The time of exposure at which all of the eggs are in that "optimum volume range" then results in 100 per cent activation.

On this basis the more rapid swelling obtained with progressively lower concentrations of sea water should cause a shifting of the activation-time curve to the left proportional to the increase in rate of swelling and likewise a shortening of the time range of activation. The results presented above show that this is in general true. But with extreme dilutions of sea water (40 per cent to distilled water) the drop to zero per cent activation does not occur. This is probably due to a secondary effect as indicated by the fact that there is a tendency for the activation to drop (see Tables II, III, IV), but as cytolysis sets in a second rise in activation (of an abnormal type) takes place.

One should also expect, according to the volume interpretation, that the concentration of sea water in which the equilibrium volume of the eggs is within the "optimum volume range" should give an activation-time curve that does not drop. This is presumably approached by the 80 per cent sea water (Table XII and Fig. 8).

3. *Activation-Volume Curves*

If the variation of the percentage of activation with time of exposure is correlated with the variation in volume of the eggs attained at corresponding times of exposure, then the percentage of activation plotted against mean volume should give a normal distribution curve, which should be identical for the various dilutions of sea water. The results show that this is roughly true. The curves obtained with various dilutions of sea water (Fig. 9) are quite symmetrical when compared with the activation-time curves. The probable reasons for the failure of the various curves to be exactly identical have been given above.

The expectation of a normal distribution curve for percentage activation plotted against mean volume is based on the assumption that the variation in volume of the eggs, at each time of exposure considered, is expressed by the normal probability curve. This is the type of variation that is generally assumed for biological material in the absence of further information. To obtain such information in this case it would be necessary to measure the volumes of a large number of eggs at various times of exposure. This has not been done on a large enough scale and accurately enough to determine whether the chance law holds for the volumes at every exposure used, but the measurements obtained on untreated eggs indicate that their variation in volume is of that type.

4. *"Over-Exposed" Unactivated Eggs*

It has been shown that the over-exposed unactivated eggs obtained with solutions ranging from 45 per cent to 65 per cent sea water can still be fertilized and may produce normal embryos. This may be taken to mean that the eggs have not been irreversibly affected by treatment with these dilutions of sea water. Consonant with this fact is the observation previously reported, that no visible changes aside from the swelling are seen to occur in the treated eggs while in the dilute sea water. It is also in accord with the result that the time for the initial stages (*e.g.* polar body extrusion) of the artificially activated eggs is comparable with that of the fertilized eggs only if allowance is made for the time of treatment.

It is evident then that no developmental changes occur in the egg while in the hypotonic solution, but that activation is initiated by the return to normal sea water after a definite time of exposure (or after a certain amount of water has been taken in). The question may therefore be raised as to why a longer exposure fails to evoke a response in the egg upon return to normal sea water when a shorter one does. If the egg were found to be injured by the longer exposure this question might be more readily answered. But the data presented here show that this is not so. The question bears directly on the mechanics of activation. With the data available we can only answer by restating the result in the following terms—that a definite change (enabling the egg to become activated upon return to normal sea water) *is* produced in the egg by the intake of an amount of water within a certain range, but that the change is reversed when more water is taken in. In other words, by the difference in behaviour upon return to normal sea water, an egg in the optimum exposure range must be intrinsically different from an egg in the earlier or later ranges, and by the similarity in behaviour upon return to normal sea water, an egg in the earlier range of exposures must be intrinsically the same (neglecting the manifest difference in volume) as an egg in the later range; hence the change produced must be reversed.

The return to original condition of eggs that have been allowed to swell in dilute sea water has also been noted in eggs of *Nereis* (Just, 1930) and eggs of *Arbacia* (McCutcheon and Lucké, 1926). But in neither of these cases is it stated whether activation is obtained at shorter exposures.

The ability of eggs that have been "over-exposed" to butyric acid to become fertilized has been noted by Moore (1916) for *Arbacia*, Just (1919) for *Echinarachnius*, and Lillie (1921) for *Strongylocentrotus*. But in these cases the cleavage and development were stated to be abnormal.

5. *Cleavage-Activation Relation*

The inverse relation between the percentage of cleavage and the total percentage of activation may now be interpreted in a similar way provided we introduce a "sub-optimum volume range" on both sides of the "optimum volume range." The justification of this arises from a consideration of the results reported in a previous publication (Tyler, 1931). It was shown that the activated eggs that extrude both polar bodies practically never divide, even though the response of that type of egg to the treatment is outwardly indistinguishable from the response of the egg to the sperm. Only the eggs that produce no polar bodies were the ones to cleave, but such eggs were shown to respond in a relatively very slow and abnormal fashion to the treatment in respect to the breakdown of the germinal vesicle, rounding out of indentation, and membrane elevation. Such is the type of result one would expect from a "sub-optimum" treatment. In terms of volume change this "sub-optimum" exposure would be obtained in a "sub-optimum volume range." Practically no eggs of that type are obtained at the time of exposure giving 100 per cent activation, but they occur in increasing numbers to either side of that exposure time. Since, at the time of exposure giving 100 per cent activation, all of the eggs are assumed to be in the "optimum volume range," the "sub-optimum volume range" must occur on each side of the former.[5]

Thus, when a batch of eggs is treated with dilute sea water, the eggs will pass through a "sub-optimum volume range" both before and after entering the "optimum volume range." At relatively short times of exposure, then, one would expect most of the activated eggs to be within the "sub-optimum volume range," and so give a high percentage of cleavage (of the activated eggs). But with longer exposures as the total activation increases one would expect more and more of the eggs to enter the "optimum volume range" and so give a low percentage of cleavage. The results would then be reversed upon passing through the second "sub-optimum volume range" with longer exposures.

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[5] The "sub-optimum volume range" must evidently be shorter than the range of variability of the volumes of the eggs, since 100 per cent cleavage (with 100 per cent activation) is never obtained for any given exposure.

This leads to a relation between percentage of cleavage and percentage total activation that is identical with that described in the text.

This interpretation can be tested in a much better fashion by following the volume changes of individual eggs in various dilutions of sea water and noting their behaviour when removed to normal sea water after having been allowed to swell to various volumes. Such experiments are now in progress.

The results reported here have an important bearing on what is generally termed the "optimum treatment" in parthenogenesis experiments. It has generally been assumed that the treatment producing the highest percentage of activation (similar to that produced by the sperm), and of cleavage and development is the optimum treatment. But in *Urechis* it has been shown that the treatment that is optimum for activation is not so for cleavage and development. Thus, if one wishes to produce the most parthenogenetic development, the length of exposure used is different from that which would be chosen if one wished to produce the highest percentage of eggs whose initial response to the treatment was most similar to that induced by the sperm. It is preferable, I think, to term the latter the optimum treatment, for the reasons stated above. The failure of eggs receiving the optimum treatment to divide is probably connected with insufficient chromatin (since all such eggs extrude two polar bodies and are left with the haploid number of chromosomes). It should be possible then to produce cleavage in such eggs by suppressing the polar divisions. This is somewhat difficult to accomplish without initiating other changes in the eggs, but the results obtained thus far indicate that suppression of the polar divisions of the "optimally" stimulated eggs results in cleavage.

The inverse relation between percentage of cleavage and percentage of activation appears then to depend on the fact that only the "poorly activated eggs" which extrude no polar bodies are the ones to divide. Thus the extent to which this relation is general for eggs of various forms will probably depend on whether or not the eggs that extrude both polar bodies divide. In eggs of the sea urchin type, where the polar bodies are extruded in the ovary and where cleavage is apparently possible with the haploid number of chromosomes, we might not expect this relation to hold.

In eggs of *Thalassema neptuni*, which, from the descriptions are very similar to the *Urechis* eggs, artificial activation by means of isotonic solutions has been reported by Hobson (1928). The variations of percentage of activation and of percentage of cleavage[6] are presented for several short series of exposures, but Hobson thinks that the results show an increase in cleavage with increase in activation. However, he notes (pp. 73 and 74) that the maximum of cleavage often fails to coincide with the maximum of activation, when both composition of medium and length of exposure are varied.

SUMMARY

1. The rate of increase in percentage activation of *Urechis* eggs with hypotonic sea water is shown to decrease as the concentration of sea water used is increased from distilled water to 80 per cent sea water.

2. The rate of increase in volume also decreases with increased concentration of sea water.

3. For dilutions of sea water ranging from 75 per cent to 45 per cent, the activation passes through a maximum (usually 100 per cent) and then returns to zero per cent with longer exposures. For lower concentrations of sea water the return to zero per cent is not obtained, but a high percentage of activation is maintained. With 80 per cent sea water the return to zero per cent activation also does not occur.

4. The activation-time curves for 75 per cent to 45 per cent sea water are of the form of skew distribution curves, rising rapidly to 100 per cent activation and falling more slowly to zero per cent.

5. The activation-volume curves are presented for 65 per cent to 45 per cent sea water and are of the form of a normal probability curve. They are roughly identical for the various dilutions of sea water.

6. Practically every series of experiments shows an inverse relation between the percentage of total activation and percentage of cleavage (of the activated eggs); so that as the percentage of activation increases with time of exposure, the percentage of cleavage decreases, and when

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[6] Hobson's total activation does not include cleavage. It is not stated in the paper whether the percentage of cleavage is that of all the eggs or of the activated eggs, though it seems to be the former. When the data of his tables is recalculated on this basis, there are thirteen cases in which an increase (or decrease) in activation is accompanied by an increase (or decrease) in cleavage and six cases in which the inverse relation holds.

the percentage of activation decreases with exposure the percentage of cleavage increases.

7. The over-exposed unactivated eggs are still capable of fertilization and of producing normal embryos in spite of the fact that a shorter exposure would have resulted in their becoming activated upon return to normal sea water.

8. The variation in rate of activation with concentration of sea water, the type of activation-time curves, the activation-volume curves, and the fertilization of over-exposed eggs are shown to be interpretable on the basis of volume change occurring in the dilute sea water, a definite volume range being optimum for activation. The cleavage-activation relation is shown to be the outcome of the previously reported result that only the "poorly activated" eggs divide, and its interpretation, based also on the exposures producing such eggs, involves the assumption of a "sub-optimum volume range" on both sides of the optimum.

BIBLIOGRAPHY

Fisher, W. K. and G. E. MacGinitie, 1928. A new Echiuroid Worm. *Ann. and Mag. Nat. Hist.*, Ser. 10, **1**: 199.

Fisher, W. K. and G. E. MacGinitie, 1928. The Natural History of an Echiuroid Worm. *Ann. and Mag. Nat. Hist.*, Ser. 10, **1**: 204.

Hobson, A. D., 1928. The Action of Isotonic Salt Solutions on the Unfertilized Eggs of Thalassema neptuni. *Brit. Jour. Exper. Biol.*, **6**: 65.

Just, E. E., 1919. The Fertilization Reaction in Echinarachnius parma. III. The nature of the activation of the egg by butyric acid. *Biol. Bull.*, **36**: 39.

Just, E. E., 1930. Hydration and Dehydration in the Living Cell. III. The fertilization capacity of Nereis eggs after exposure to hypotonic sea water *Protoplasma*, **10**: 24.

Lillie, F. R., 1921. Studies of Fertilization. IX. On the question of superposition of fertilization on parthenogenesis in Strongylocentrotus purpuratus. *Biol. Bull.*, **40**: 23.

McCutcheon, M., and Lucké, B., 1926. The Kinetics of Osmotic Swelling in Living Cells. *Jour. Gen. Physiol.*, **9**: 697.

Moore, C. R., 1916. On the Superposition of Fertilization on Parthenogenesis. *Biol. Bull.*, **31**: 137.

Tyler, Albert, 1931. The Production of Normal Embryos by Artificial Parthenogenesis in the Echiuroid, Urechis caupo. *Biol. Bull.*, **60**: 187.

THE OCCURRENCE OF MELANOPHORES IN CERTAIN EXPERIMENTAL WOUNDS OF THE GOLDFISH (*CARASSIUS AURATUS*)[1]

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While studying in the goldfish the repair of experimental wounds, crushes, burns, and fractures, it became apparent that melanophores developed in the wounds a few days after the trauma and later degenerated and thus disappeared. Not alone did these melanophores occur directly at the site of the injury, but not infrequently in the corium of adjacent areas and even in remote cutaneous regions. In none of these places were black pigmented cells seen by a previous low-power microscopic examination of the living fishes used for the experiment, nor were melanophores of the corium noticeable by high magnification in sections of tissue removed from the region of the wound at the time of trauma. The appearance of pigmented cells at the very point of injury seemed to indicate a rôle of importance for melanophores of this fish, from the viewpoint that these cells functioned in the processes of repair and, not unlikely, in the mechanism of body defense.

As results of different experiments were found to be uniform, only a few are here reported in detail as illustrative.

Experiment 1. Goldfish, 8 cm. long from snout to base of tail, kept in still water tank, supplied by current of air. Temperature of water 78° F.

Oct. 28, 1930. Transverse incision was made with a cataract knife through a single ray of caudal fin, near the upper edge of middle part of this fin. Incision penetrated tissues over both surfaces of fractured ray.

Oct. 30. Overlying the ray near the fracture are a few scattered melanophores with irregular processes (Fig. 1). Tissues overlying the fracture are œdematous and difficult to photograph for this reason. There are a few small points of hemorrhage near the fractured fragments.

Oct. 31. A large number of melanophores, in places interlacing, surround the proximal and the distal fragment of the fractured ray as if to encapsulate the fragments (Fig. 2).

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[1] Aided by grant from Blossom Fund.

Nov. 1. Active degeneration of melanophores has begun with pigment granules lying free in tissue spaces (Fig. 3).

Nov. 5. Degeneration of all melanophores in the region of the fracture, with many small pigment masses scattered throughout the field.

Nov. 8. Entire region of fracture, somewhat whitish and translucent, shows no more evidence of pigment.

In the following experiment multiple injuries were produced.

Experiment 2. Two goldfishes, 7 cm. from snout to base of tail were placed in a tank of still water fed with a current of air. The temperature of the water was gradually raised from 70° F. by heating over a period of three days to 84° F.

Sept. 23, 1930. In both fishes eight different regions were clamped with an artery forceps each for 15 seconds. The points clamped were as follows: right and left operculum, both pectoral, both ventral, the anal and the caudal fins.

Sept. 25, 2 P.M. One fish shows early pigmentation by melanophores in caudal fin, the second fish has melanophores in the right ventral fin. Pigmentation is slightly distal to crush.

Sept. 26, 3 P.M. Three days after trauma, both fishes show pigmentation by melanophores at all eight points crushed. The pigmentation is a marked one due to the large number of melanophores present in the crushed zones and neighboring tissue.

Explanation of Plate 1

Figs. 1–4. Experimental linear fracture by incision of a ray of caudal fin of goldfish, the injury including all tissues directly overlying fracture. Letters *A* and *B* indicate site of fracture. All photomicrographs taken from the same living fish anæsthetised with chloretone 1–2000. Magnification × 90. Temperature of water 78°–80° F.

Fig. 1. Two days after injury. A few melanophores have appeared in the œdematous tissue near the fracture, *A.B.*

Fig. 2. Three days after injury. Numerous melanophores appearing as single cells or interlacing cells at the line of fracture, *A.B.*

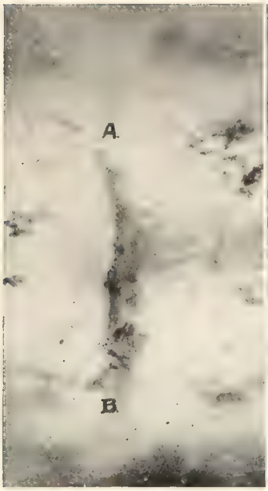
Fig. 3. Four days after injury. Degeneration of melanophores at the site of fracture *A.B.* has begun. Small black pigment masses from degenerated cells lie scattered among living melanophores.

Fig. 4. Five days after injury. Degeneration of melanophores at the site of fracture *A.B.* is complete, scattered pigment debris remains in the field. Final disappearance of all pigment on the eighth day after injury.

Fig. 5. Inter-radial tissue of caudal fin showing melanophores distributed near capillaries marked *A*, *B*, *C*, *D*. × 60. Fresh tissue removed from goldfish near an area crushed eight days previously. Fish outdoors exposed to sunlight,

Fig. 6. Irregular areas of pigmentation of melanophores developing on the surface of the body of a goldfish injured by removal of all body scales five days previously. Photograph made from living fish anæsthetised with chloretone. Size, two-thirds normal.

Plate I



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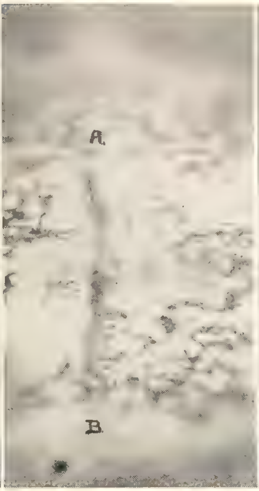

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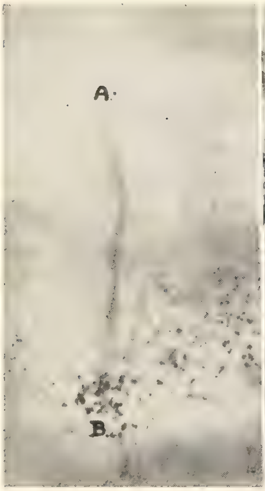

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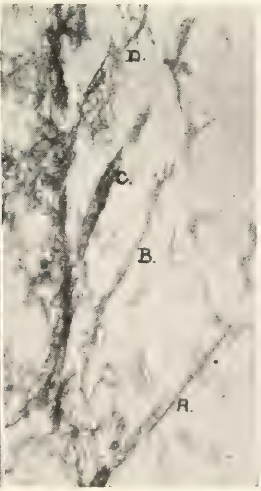

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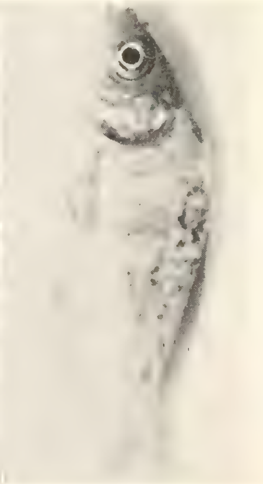

6.

Sept. 28. There is evidence of degeneration of melanophores at all crushed points. Temperature, 90° F.

Oct. 5. One fish is entirely clear of degenerated pigment granules at crushed point. The second shows a few black granules in the wound of the caudal fin.

Oct. 6. In both fishes all evidence of pigment formed of degenerated melanophores has disappeared at all eight points crushed. Thus these two fishes injured by crushing at eight separate points have shown, with temperature of water between 84° F. and 90° F., an intense pigmentation by melanophores at crushed points, a subsequent degeneration of melanophores, and a complete disappearance of all pigment detritus all in the course of 13 days.

Experiment 3. In this experiment, involving injury to the right operculum, 30 goldfishes, about seven cm. in length, were used. These were divided into three groups of ten. Each group was placed in a separate tank of running water in the laboratory. Fishes in Tank 1 were operated on by resecting one third of the right operculum by a straight vertical cut with scissors. Fishes in Tank 2 received a simple vertical crush for fifteen seconds of the middle of the right operculum. Fishes in Tank 3 were first crushed for 15 seconds by a clamp placed vertically in the mid-point of the operculum and all opercular tissue distal to the clamp was resected. Tank 1—Fishes (simple excision of one half of the right operculum) showed melanophores in the margin of the wound three days after operation. At first only a few such cells, but in the following two or three days there were many present. Evidence of degeneration of melanophores was noted in places as early as two days after their first appearance. Complete disappearance of black degenerated pigment from the wounded area varied between 3 and 9 days. Fishes in Tanks 2 and 3 with more severe injuries of the operculum showed a beginning accumulation of melanophores in the injured operculum also three days after trauma. The entire disappearance of pigment from the wound in fishes in Tank 2 (vertical crush of operculum) varied between 6 to 15 days after appearance of melanophores. In Tank 3 (fishes with crushed and partially resected right operculum) the eruption of melanophores at the injury occurred also three days after injury, but the final disappearance of pigmented debris varied between 9 and 16 days. One fish in Tank 1 and four fishes in Tank 2 showed slight pigmentation by melanophores of the opposite uninjured operculum, arising when the accumulation of melanophores on the injured side was well developed. Melanophores in the area of secondary pigmentation degenerated and disappeared before those in the experimentally injured right operculum.

SUMMARY

The onset of cutaneous pigmentation by melanophores in three different types of wound of the operculum carried on simultaneously in three different tanks of running water at 76° F. was uniformly between the third and fourth day after trauma. The final disappearance of pigment of degenerated melanophores of the wound area varied between 6 and 19 days after injury. In some fishes the accumulation of melanophores noted at the wound was relatively slight; in others the black pigmentation caused by large numbers of melanophores was intense and remained over a longer period.

Fishes operated on during the cold winter months and kept in tanks of cold running water (43° F.) did not show at wounded areas such a rapid development of melanophores as described in the preceding experiment. Further, pigmentation of wounds under winter temperature extended over longer periods. Thus, in nine fishes with right operculum crushed for 15 seconds with an artery clamp placed at the middle of the operculum, followed by excision of opercular tissue distal to the operculum, the following results were obtained: An eruption at the injured operculum in all nine fishes occurred between 13 to 16 days after injury; pigmentation had cleared up by degeneration of melanophores in only three fishes two months after injury, with temperature of water at 53° F. It took approximately one more month (temperature 53°–56° F.) for four more fishes to clear; the remaining two fishes cleared at the end of still another month or four months from the date of injury, when the temperature of the water had gradually risen to 61° F. The longest period of pigmentation in a wound of this series represented approximately 110 days from the date of the first appearance of melanophores.

It became of interest to learn whether or not in fishes kept in very cold water, an appearance of melanophores after trauma could be temporarily inhibited, to appear for the first time when such fishes were changed back slowly to more favorable warmer temperatures. A number of experiments were done along these lines.

Experiment 4. A goldfish, seven cm. in length, was placed in a tank of still cold water supplied by a current of air, the water varying in temperature between 42° F. and 45° F. The tank was set up in a refrigerator arranged with a double window, admitting ample daylight. It was found advisable to accustom the experimental fishes gradually to cold. By using several submerged electric lights at the beginning of the experiment and turning these off as desired, the temperature of the water could be lowered slowly without endangering the life of the fish.

Oct. 14, 1930. A small incision was made with a cataract knife in the caudal fin of this goldfish dividing transversely a single ray near the upper margin of the fin. Examination of melanophores at four day intervals negative for an entire month. Temperature 42° to 46° F.

Nov. 14. Temperature in tank raised slowly so as to reach 66° F. on Nov. 16th.

Nov. 16. Numerous melanophores appeared for the first time at fracture and along injured ray distal to this. No other black pigmentation noted.

Nov. 25. Slight pigmentation by melanophores of tip of tail and also along the margin of dorsal fin. Large accumulation of melanophores at fracture.

Nov. 27. Active degeneration of melanophores at fracture and other pigmented regions.

Nov. 29. Fish under dissecting microscope shows no pigment masses either at site of experimentally fractured ray or at the secondary points of black pigmentation of tail or dorsal fin. All melanophores have disappeared by a process of degeneration.

Fishes kept in a dark chamber, excluding all light, developed melanophores in wounds as promptly as did controls kept in daylight.

Experiment 5. Two goldfishes, seven cm. in length, with crushed right operculum and caudal fin, kept in a dark chamber in a tank of still water at 64° F., supplied by air current, were taken out of this chamber to be examined for the first time after injury on the fifth day. Many melanophores were present in crushed regions. At the same time, two control fishes, injured on the same day in a similar way, kept in a tank of equal size at the same temperature but exposed to laboratory daylight, exhibited, also for the first time, a large number of melanophores at the two crushed points. Twenty-three days after injury, one fish contained in the dark chamber and both controls were clear of pigment; the second fish in the dark chamber showed no melanophores in the injured operculum, although a few small masses of degenerated pigment masses still remained in the caudal fin.

The production of a second injury in a healed wound frequently, but not always, caused another eruption of melanophores. Refracturing a single ray at the same point, especially where the previous healing had left a wide whitish translucent area, did not produce a second crop of melanophores. The very simple injury of making a longitudinal slit in the caudal fin did not call forth melanophores either at the time of the first injury or with repeated incisions at the same point.

The irregular topographic distribution of melanophores following trauma was seen particularly well in experiments where the scales on both sides of the body were totally removed.

Experiment 6. Nov. 1930. Three goldfishes, *A*, *B*, *C*, measuring 8, 7, 5 cm. in length respectively, kept in a heated tank of still water 76° F., supplied by air current, were operated on under chloretone anæsthesia (1–2000). All scales of the body were removed with forceps in all three fishes.

Dec. Four days after operation, melanophores appeared in irregular groups at various points on both sides of the body. The two larger fishes, *A* and *B*, showed in the course of the next few days a large number of melanophores in irregular scattered patches. The patches of pigmentation by melanophores in fish *B* are shown in Fig. 6. The smallest fish, *C*, showed only a few melanophores in small, widely-scattered areas. By the end of the twelfth day degeneration of melanophores evoked by removal of scales had occurred in all three fishes with a disappearance of broken-down pigment material. At this time (12 days after removal of scales) each fish showed definitely a set of new young scales. Fish *B* successfully withstood a second complete removal of scales, under chloretone anæsthesia, but this time only a very few rapidly degenerating melanophores developed on the denuded surface of the body, as if the supply of pigment-forming cells for these particular surface areas were partially exhausted. When, however, on the fourth day after the second operation for removal of scales the caudal fin of this fish was crushed by clamp for 15 seconds, numerous melanophores developed three days later in the crushed tail but in no other place.

DISCUSSION AND SUMMARY

Various important problems relating to melanophores and melanogenesis appear in connection with the works of Van Rynberk (1906), von Frisch (1911), Weidenreich (1912), Asvadourova (1913), Spaeth (1913), R. Fuchs (1914), Wyman (1924), Wells (1925), Abolin (1925), Ewing (1926), Jost (1926), Bloch (1927), Cordier (1928), Becker (1930).

For the present purpose it may be of interest to recall that a number of years ago Weidenreich (1912) showed that in vertebrates the distribution of black pigment cells could be regarded as forming four distinct envelopes for the body. These envelopes he designated as "cutaneous, perineural, pericoelomatic and perivascular" respectively. He pointed out that whereas in some vertebrates several or all of these pigmentary envelopes were well developed, in other vertebrates one or more of these pigmentary envelopes might be found poorly developed, showing only a trace or rudiment of pigmented tissue. For example, in man, where there exists a well developed cutaneous envelope of pigmented tissue, the perineural pigmented tissue is poorly developed, presenting itself as scattered black pigment cells of the piamater and elsewhere in the brain. In fishes all pigmentary envelopes are regarded as fairly well developed.

In interpreting the meaning of melanophores following injury as seen in the above experiments on goldfish, it should be kept in mind that such melanophores may represent a perivascular or perineural type of cell developing the properties of forming pigment, rather than cells belonging strictly to a system of cutaneous melanophores. It is particularly the cutaneous or corial melanophores of fishes which have received the most study to date.

Melanophores, according to Bloch (1927) show a number of morphologic peculiarities in that they form processes or dendrites and have a tendency to arrange themselves in an interlacing network. They exhibit in cold-blooded animals certain functional reactions which are shown by the spreading or the contraction of the intracellular masses of pigment granules. These reactions are changes which have their origin in nervous, actinic or hormonal stimuli; and they may also be produced by mechanical, chemical and electric means.

Ever since the description of melanophores in fishes by Siebold (1861), many investigators have contributed to the morphology of this subject. The works of Ballowitz (1912–16) on the different types of chromatophores (*i.e.*, the melanophores, xantho or erythrophores, guanophores and their various combinations forming what he designated as chromatic organs) have largely laid the basis for our present knowledge of pigment cells in fishes. This author also demonstrated histologically the innervation of melanophores in fishes.

The experimental observations of Pouchet (1876) showed a relationship between cutaneous melanophores in fishes and the sympathetic nervous system. It remained, however, for von Frisch (1911, 1912), in a series of important experiments, to demonstrate in fishes a contraction center for cutaneous melanophores in the front part of the medulla, and a secondary center in the spinal cord. Further, he explained the pathways by which impulses pass from brain through pigment motor nerve fibers to the sympathetic system and from here by means of the peripheral nerves not only to the melanophores but also to other chromatophores of the skin.

In general, the function of melanophores has been variously interpreted. In addition to the view that cutaneous pigmentation and pigment changes represent color adaptation to environment, the purpose of cutaneous pigment has been thought to lie in its protection of deeper tissues against injurious solar rays. The migration of retinal pigment granules as it applies to vertebrates and arthropods is thought by Parker (1906) to be a mechanism calculated to protect the receptive organs of the retina from overstimulation by light and to improve the retinal images. Cutaneous pigment cells have been regarded as transforming light into heat energy. According to this view, as Weidenreich (1912) explains, the minute individual intracellular pigment granules of melanophores become heat bodies or Heizkörper, which distribute heat to neighboring protoplasm. Weidenreich (1912) has further suggested, because melanophores are innervated and react to optic, thermic and chromatic stimuli, that they may be regarded perhaps as sensory cells for color and warmth perception.

Cordier (1928) believes that the formation in cells of melanin is a process of excretion as yet not well understood. The theory implies that certain toxic waste products of metabolism gain access to special cells and there become insoluble and pigmented, their toxic products being neutralized. Elimination of pigment follows slowly as if it were a process of retarded excretion. Certain clinical cases of Addison's disease and melanosarcoma have shown melanin greatly increased in cutaneous areas and present in the blood and in the urine. This has been taken to mean a profound chemical disturbance of the body as a whole and gives support to the view that a general metabolic process may ordinarily affect the production of melanin in various regions of the body.

Whatever may be the relationship to the nervous system of melanophores resulting from trauma as seen in the present experiments on goldfish, it seems plausible from their structural arrangement in healing wounds, that such melanophores are pigmented cells which function in repair of damaged tissue. Melanophores of this kind appeared relatively early in the course of wound-healing when favorable warm temperatures were employed. They disappeared by a process of degeneration at the site of the wound when healing proceeded and usually when the covering of the wounded surface was nearing its completion. Whereas melanophores showed in wounds of goldfishes within 3 or 4 days after injury when fishes were kept in water of relatively warm temperature (70°–90° F.), with fishes kept in cold water (40°–42° F.) the appearance of melanophores in wounds was retarded or even inhibited, to appear for the first time when these fishes were returned to a warm environment. A temperature of 40° F. was found sufficient to inhibit the appearance of melanophores for a month.

Fishes kept in a dark chamber completely excluding light showed melanophores in various experimental wounds as early as did controls kept under usual laboratory conditions exposed to light. Fishes kept in tanks out-of-doors and in this way exposed directly to the sunlight developed melanophores in wounds a few days later (Fig. 5). The reaction here seemed intense. In some of these fishes melanophores developed not alone at the crushed points, but also in areas adjacent to the wound and in all other fins.

When studied in a simple form of injury such as dividing transversely a single ray of the caudal fin, melanophores appeared first as periadventitial cells in close relation to the outer walls of the small capillary blood vessels which covered the surface of the ray near the fracture. With an increase in numbers, the melanophores spread toward the region of the fracture and formed a network (Fig. 2) in the corium by the interlacing of the numerous irregular processes. Degeneration in individual melanophores was observed as early as 24 hours after their first appearance near a fractured ray. Fixed paraffin sections of tissue with degenerating melanophores showed a moderate number of phagocytic cells containing pigment. For the most part, however, the impression was gained that the pigment detritus rested free in the tissue spaces preparatory to removal by lymphatics, or became dissolved *in situ*.

The actual production of melanin in cells is now generally regarded as the result of enzyme action. The important studies of Bloch (1927), advancing the views on the intracellular production of melanin by enzyme, are too well known to need repetition here. It is conceivable that in the experimental wounds of goldfish chemical changes occur locally permitting melanin to be formed in periadventitial cells irregularly distributed in the corium of the injured area.

Experimental wounds of goldfishes quite naturally are constantly open to infection by bacteria or parasites. Numerous bacteria and especially cocci were seen in paraffin sections of tissue from crushed operculum at various stages after injury before complete healing had occurred. When, as occasionally noted, a growth of fungus appeared in connection with experimental wounds, pigmentation by melanophores appeared particularly intense, affecting not alone the wound but also adjacent areas. There was at times pigmentation of the fins other than the ones experimentally injured and, in rare instances, a patchy pigmentation of body scales under these circumstances. Treating such wounds for several days in succession with two per cent mercurochrome destroyed the parasites, and pigmentation of the wound with secondary pigmented areas then disappeared. The presence of bacteria in wounds and the large number of melanophores present in injured areas affected with parasites, suggest a possible rôle for melanophores in the mechanism of body defense.

Goldfishes subjected to a total removal of scales showed in the course of several days a distribution of melanophores varying in extent and intensity in different fishes. This eruption was asymmetrical, irregular and patchy, as if periadventitial cells capable of forming black pigment as a result of trauma or during subsequent wound regeneration, actually occupied a very irregular distribution on both sides of the body. As new scales formed in these experimentally produced scaleless fishes, melanophores disappeared by degeneration. A second total removal of scales in one of the fishes was followed by a very scanty eruption of melanophores, as if the possibility of local melanophore production in this instance were, temporarily, at least, exhausted.

Usually, but not always, a re-injury at the same point brought out a second eruption of melanophores differing but little from that which followed the primary injury.

The eruption of melanophores in experimental wounds of the goldfishes, varying in intensity in different fishes, appears to indicate that such melanophores, probably periadventitial in origin, form in response to injury and function in the repair of injured tissues.

LITERATURE CITED

ABOLIN, L., 1925. Beeinflussung des Fischfarbenwechsels durch Chemikalien. *Arch. f. mikr. Anat. und Entwick.*, **104**: 667.

ASVADOUROVA, N., 1913. Recherches sur la formation de quelques cellules pigmentaires et des pigments. *Arch. d'anat. micros.*, **15**: 153.

BALLOWITZ, E., 1893. Die Nervenendigungen der Pigmentzellen, ein Beitrag zur Kenntnis des Zusammenhanges der Endverzweigungen der Nerven mit dem Protoplasma der Zellen. *Zeitschr. f. wissenschaft. Zool.*, **56**: 673.

BECKER, S. W., 1930. Cutaneous Melanoma: a Histologic Study especially directed toward the Study of Melanoblasts. *Arch. Dermat. and Syph.*, **21**: 818.

BLOCH, B., 1927. Das Pigment. Handbuch d. Haut. u. Geschlechtskrankheiten Berlin, Vol. 1, Part 1, pp, 434–541.

CORDIER, R., 1928. Les pigments mélaniques et la mélanogénèse. *Bull. Soc. Roy. d. Sc. med. e nat. de Bruxelles*, Nos. 2–7, pp. 43–57.

EWING, J., 1922. Neoplastic Diseases. Philadelphia, pp. 871–890.

VON FRISCH, K., 1911. Beiträge zur Physiologie der Pigmentzellen in der Fischhaut. *Arch. f. ges. Physiol.*, **138**: 319.

VON FRISCH, K., 1912. Über farbige Anpassung bei Fischen. *Zool. Jahrbuch*, **32**: 171.

FUCHS, R. F., 1914. Der Farbenwechsel und die chromatische Hautfunktion der Tiere. *Handbuch d. vergleich. Phys.*, **3**: 1189.

JOST, F., 1926. Die Farbzellen und Farbzellvereinigungen in der Haut des Nordseefisches Callionymus lyra L. *Zeitschr. f. mikr. anat. Forschung*, **7**: 461.

PARKER, G. H., 1906. The Influence of Light and Heat on the Movement of the Melanophore Pigment, especially in Lizards. *Jour. Exper. Zoöl.*, **3**: 401.

POUCHET, G., 1876. Des Changements de coloration sous l'influence des nerfs. *Jour. de l'Anat. et de Physiol.*, **12**: 1–90, continued 113–165.

VON SIEBOLD, C., 1863. Die Süsswasserfische von Mitteleuropa. Leipzig, p. 14.

SPAETH, R. A., 1913. The Physiology of the Chromatophores of Fishes. *Jour. Exper. Zoöl.*, **15**: 527.

van Rynberk, G., 1906. Über den durch Chromatophoren bedingten Farbenwechsel der Tiere (sog. chromatsche Hautfunktion). *Ergebn. der Physiol.*, **5**: 347.

Weidenreich, F., 1912. Die Lokalisation des Pigmentes und ihre Bedeutung in Ontogenie und Phylogenie der Wirbeltiere. *Zeitschr. f. Morph. u. Anthrop.*, Sonderheft 2, pp. 59–140.

Wells, H. G., 1925. Chemical Pathology. Philadelphia, pp. 526–532.

Wyman, L. C., 1924. Blood and Nerve as controlling Agents in the Movements of Melanophores. *Jour. Exper. Zoöl.*, **39**: 73.

STUDIES ON THE INTESTINAL FLORA OF TERMITES WITH REFERENCE TO THEIR ABILITY TO DIGEST CELLULOSE

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Introduction

Interest for a considerable time has been centered on the ability of certain organisms to derive nourishment from a wood diet, the principal constituents of which are cellulose and lignin, both resistant to the digestive action of enzymes normally present in the digestive tract of most animals. Animals such as termites, larvæ of wood-boring beetles, and a bivalve, the shipworm *Teredo navalis*, so injurious to ships and piles, have been conspicuous for their ability to digest cellulose. In the case of *Teredo navalis* (Dore and Miller, 1923), digestion has been explained by the production of cellulose-digesting enzymes. The explanation in the case of termites is a more interesting one.

Microscopic examination of the intestinal content of most species of termites discloses countless numbers of Protozoa and bacteria. Careful experiments have been carried out to explore the relationships between the host and their intestinal organisms, and it has been shown conclusively that the termites are absolutely dependent upon the Protozoa present for the digestion of the cellulose in their food.

L. R. Cleveland, one of the foremost workers in this field, has shown that termites containing an intestinal fauna of Protozoa were able, under favorable conditions, to exist on a diet entirely made up of Whatman filter paper of the purest grade, and he successfully reared them upon this diet for over two years. If, however, he defaunated the termites (by incubating them at 36° C., the Protozoa were killed, but the termites were unharmed), they were unable to live upon the pure cellulose and soon died. If he re-inoculated the termites with Protozoa, however, after incubation, they were able to live indefinitely upon the filter paper (Cleveland, 1924).

Biological Considerations

Comparatively little work has been done with respect to the biological relationship between termites and the other intestinal organisms usually present. Hollande (1922) discusses the morphology and reproduction in a considerable number of spirochætes which he found very abundant in the intestinal contents. Hoelling (1910), in his paper on "The Nuclear Conditions of Fusiformis termitidis," describes the morphology of fusiform bacilli studied from a number of smears of the intestinal content of termites. Imms (1924) states that "Portier investigated an apparent symbiosis in the case of the larva of *Nonagria* which lives within the stems of *Typha* devouring the pith. . . . In the digestive tube of this larva are found great numbers of motile conidia of a fungus (*Isaria*), which exist among the devoured vegetable fragments. The conidia are always accompanied by a micrococcus which secretes an enzyme capable of dissolving cellulose. Portier states that the conidia develop at the expense of the dissolved cellulose and eventually penetrate the walls of the gut, escaping into the blood. Most of them are there attacked by phagocytes and transformed into products which serve to nourish the tissue of the host."

In an attempt to determine whether the Protozoa were entirely responsible for the digestion of cellulose in the digestive tract of termites, Cleveland (1924) studied the bacterial flora of *Reticulitermes flavipes*. He states that bacteria were sometimes numerous, and he attempted all known methods, aërobic and anaërobic, for isolating cellulose-digesting bacteria. One hundred attempts were made, but all results were negative, even after the cultures were more than two months old. Ten attempts to isolate cellulose-decomposing molds and actinomycetes were made and were unsuccessful. In an attempt to isolate the organisms, an inorganic medium was made containing:

K_2HPO_4	1.00 gram
$MgSO_4$	0.50 gram
KCl	0.50 gram
$FeSO_4$	.01 gram
$NaNO_3$	2.00 grams
H_2O	1000 cc.

To this medium cellulose was added in two forms: a small piece of Whatman's filter paper and 0.5 per cent cellulose suspension. To the inorganic medium containing cellulose suspension sufficient agar was added to make a solid medium. Incubation was apparently 36° C.

Cleveland (1928) observed that all families of termites harbored many spirochætes which he thought might play a rôle in the digestion of cellulose and hemicellulose. He observed millions of these, often attached to a single Protozoön, and easily mistaken for flagella. Attempts to grow the spirochætes failed and animal inoculations proved negative. By feeding the termites cellulose thoroughly moistened with a 5 per cent aqueous solution of acid fuchsin, he found it possible to remove in this manner all spirochætes without doing any damage whatever to the Protozoa or to the termites. He concluded that the spirochætes play little if any rôle in the digestion of wood and cellulose.

In the wood-ingesting larvæ of certain insects, characteristic blind sacs and diverticula of the digestive tract have been demonstrated in which, aided by the action of myriads of bacteria, food particles are held and digested. From the larvæ of rose-chafers (*Potosia cuprea*) cellulose-digesting bacteria have been isolated in pure culture. These slender peritrichiate, anaërobic rods (*Bacillus cellulosae fermentans* Werner) are found free also in the ant hills inhabited by *Potosia cuprea*. The optimum temperature for fermentation was found to be 33–37° C., the minimum 21° C. The larvæ are so dependent upon these intestinal organisms that the increase in weight of the larvæ is determined by the temperature. If the temperature of the ant hill at the end of October goes below 21° C., the now useless taking-in of food material is suspended (Buchner, 1928).

EXPERIMENTAL

a. The Food of Termites

It was the purpose of the present experiment to determine whether cellulose-digesting organisms occurred in woody material upon which termites feed. The woody material from termite colonies was first examined for cellulose-digesting organisms. Some material obtained from a termite colony in March, 1929, which had been kept in a dry condition since that time (almost one year), was used to inoculate nitrate-cellulose tubes. The material contained wood particles, termite excreta and a small quantity of fine reddish clay.

The nitrate-cellulose medium was made according to the formula of Bradley and Rettger (1927). It contained:

Di Potassium phosphate	1 gram
Magnesium sulfate	1 gram
Sodium chloride	1 gram
Calcium carbonate	2 grams
Potassium nitrate	2 grams
Distilled water	1000 cc.

The cellulose was provided in the form of strips of filter paper. Tubes were kept at room temperature and incubated at 34.5° C. and 60.5° C. under aërobic and anaërobic conditions.

Ten tubes were incubated at room temperature under aërobic conditions. In every one of these there was abundant growth and discoloration of paper above the liquid level. Marked discoloration appeared in all tubes in four days. Seven tubes labeled G–1 to G–7 inclusive were inoculated from two of the above tubes on March 1, 1930. When examined on March 3, 1930 discolored areas were present on the paper of all tubes. On the original tubes the discolorations were yellow-green, yellow and light brown. On the "G" tubes the predominant color was dark brown.

Three tubes inoculated and placed under anaërobic conditions on February 19, when examined on March 8, showed no marked discoloration and no cutting of paper at this time. (Anaërobic conditions were produced in a Mason jar, using pyrogallic acid and sodium hydroxide.) On this date the three tubes were placed under aërobic conditions. When examined April 7, tube no. 16 showed cutting of paper at liquid level and maceration of paper below liquid level. Mold growth apparently was inhibited.

A tube no. 12 incubated at 34.5° C. under aërobic conditions on February 19, when examined on February 25 showed marked discoloration similar in variety and extent to that produced at room temperature. The paper, however, was not cut at liquid level.

Three tubes 17, 18, and 19, inoculated and incubated at 34.5° C. under anaërobic conditions, showed no noticeable discoloration or cutting of paper when examined 18 days later. The tubes were removed from the incubator and placed under aërobic conditions, at room temperature. When examined 16 days later, the paper in tubes 18 and 19 was entirely cut at liquid level with no characteristic discoloration. The paper below the liquid level was entirely macerated. (The paper was probably cut before the day of examination.)

A tube incubated at 60.5° C. under aërobic conditions showed, when examined about 10 days later, a few small, isolated areas of growth on paper.

Three tubes were inoculated and incubated at 60.5° C. under anaërobic conditions on February 20. When examined on March 7, one of the tubes showed paper cut at liquid level and macerated at lower portion below liquid level. In this tube the paper was so macerated that upon slight shaking it fell apart into loose fibers below liquid level.

From this last tube three tubes were inoculated and incubated at 60.5° C. under aërobic conditions on March 8, three silica-gel plates inoculated from the above tube also, were incubated anaërobically at 60.5° C. On April 7, neither plates nor tubes showed discoloration or growth. Re-inoculated nitrate-cellulose tubes at 60.5° C. under anaërobic conditions showed no growth after one month.

Woody material obtained with termites from Dr. Cory was used to inoculate a tube at room temperature under aërobic conditions. The material was composed of wood and digested material, but no loose soil. When examined 9 days later the paper at the liquid level showed a discoloration which was marked above liquid level. Green discoloration was prominent. One colony 0.5 mm. in diameter produced a wine-colored discoloration. Transplants on silica-gel plates produced abundant growth.

MARINE BIOLOGICAL
WOODS HOL

The silica-gel was made fundamentally according to the general formula of Winogradsky (1929). Seventy-five grams of potassium silicate were dissolved in 1000 cc. of distilled water. To this was added an equal quantity of HCl of a specific gravity of 1.10. Thirty cc. of mixture were placed in the petri dishes and set aside under cover for 24 hours. They were then immersed in running water for 24 hours. They were next washed four successive times for 24 hours each in large covered dishes of sterile distilled water. From the following inorganic salt solution (made up for 100 plates) 2 cc. were added to each silica-gel dish.

KH_2PO_4	1.0 gram
$MgSO_4$	0.5 gram
NaCl	0.5 gram
$FeSO_4$	0.01 gram
$MnSO_4$	0.01 gram
KNO_3	3.6 grams
$CaCO_3$	2.0 grams
Distilled water	200 cc.

The pH was adjusted to 7.2. The petri dishes were then placed in an incubator at 60.5° C. until excess moisture evaporated. Sterilized pieces of Whatman's filter paper were placed aseptically on silica-gel plates. Sterile covers were then placed over the plates. Stroke inoculations were made.

Termites received from Dr. Cory were transferred to clean petri dishes in which were placed filter paper and the tissue paper sent with the original shipment. This was being eaten by the termites. On March 8, pellets of termite excreta which had been dropped on the tissue paper were used to inoculate two nitrate-cellulose tubes and one silica-gel plate, and kept at room temperature. (The pellets were clean and the color of tissue paper.) On March 10 no visible growth was evident in the tubes. Yellow discoloration was noticeable on the paper around the pellets in the silica-gel plates. This growth later covered the entire paper and the paper showed almost entire digestion by May 10. Nitrate-cellulose tubes inoculated from this plate showed growth and digestion of paper in 5 days. A gelatinous milky-white growth with translucent areas developed on paper. Microscopic examination showed maceration of fibers with attached bacteria. The growth was characterized by the presence of mold growth which formed the gelatinous milky mass.

b. The Intestinal Contents of Termites

Most of the experiments with the intestinal contents were carried out with termites of the genus *Reticulitermes* collected at Mullica Hill, New Jersey, and with *Termopsis*, received from Dr. Kirby, at the University of California.

Reticulitermes.—Microscopic examination of intestinal contents showed besides the myriads of Protozoa, spirochætes ranging in length from 5 to 15 μ; the smaller ones were in great abundance, and apparently more numerous than the Protozoa. Motile rods and filamentous rods were also present. Examinations were made with hanging drops in physiological salt solution and from smears stained with alcoholic fuchsin.

On March 31, seven inoculations were made with intestinal contents of seven termites, washed 4 minutes in 1–1000 $HgCl_2$, then rinsed with sterile distilled water. The intestinal contents were squeezed out with sterile forceps onto silica-gel plates. These were kept anaërobically at room temperature. On April 1, sixteen termites were used as above to inoculate silica-gel plates. Eight of these were kept under anaërobic conditions and eight were kept at room temperature under aërobic conditions. On April 2, twenty termites were used as above to inoculate silica-gel plates kept at room temperature under aërobic conditions. On April 1, six termites were used to inoculate six nitrate-cellulose tubes.

When the above cultures were examined on May 12, none of the anaërobic plates showed growth. Of the aërobic silica-gel plates eight inoculations showed a slight mold growth, with no cutting of paper and no distinct discoloration. The remaining inoculations on plates produced no growth. One of the six nitrate-cellulose tubes showed clearly digestion of paper, with translucent areas, and microscopic examination of paper from this tube showed numerous bacteria, $1 \times 0.75\ \mu$ in size on the fibers.

Four tubes inoculated on February 28 with *Reticulitermes flavipes* received from Dr. L. R. Cleveland showed no growth on March 10, and no growth was visible on April 30.

Termopsis.—The specimens were large enough to enable one to dissect out the digestive tract with sterile forceps. All termites were first washed for 5 minutes in 1–1000 $HgCl_2$ and then rinsed in sterile distilled water. All inoculations were made on silica-gel plates, which were kept under aërobic conditions at room temperature.

On April 21, eight termites were used for inoculations. On April 23, two winged termites were used to inoculate silica-gel plates. On April 24, ten winged termites were used for inoculations. On April 24, eight worker termites were used as above. On May 2, six workers were used as above. On May 2, eight winged forms were used as above. On April 23, ten termites were used to inoculate silica-gel plates which were kept under anaërobic conditions. When examined on May 17, none of the plates showed cellulose-digestion or indication of growth of cellulose-digesting organisms. There was mold growth on intestinal contents of eighteen termites. The growth of molds was evidently due to the fact that treatment for 5 minutes with 1–1000 $HgCl_2$ did not kill them.

Beckwith and Rose (1929) obtained cellulose digestion in a number of cases when working with intestinal contents of termites, but their results cannot be taken as conclusive, since they attempted to sterilize the termites externally by merely washing them in tincture of iodine (U.S.P.) for 45 seconds. This short exposure and the fact that small air bubbles captured between the hairs of the insect would prevent contact with the germicide in this time, would indicate that the organisms on the surface were not destroyed.

Discussion of Results

Cleveland and others have shown that digestion of cellulose in termites is entirely dependent upon intestinal organisms. Cleveland has shown that termites are not dependent upon intestinal spirochætes, although he has not shown that the spirochætes do not play an important part in cellulose digestion.

In every experiment carried out in the present study with woody material from termite nests, abundant growths of cellulose-digesting organisms were obtained. The numerous cellulose-digesting organisms, which were undoubtedly taken into the digestive tract with food, could not again be isolated from the intestine on the cellulose media used.

The most conspicuous organisms, with reference to numbers and bulk next to the Protozoa, as seen by microscopical examination of the intestinal contents of termites, are the spirochætes. Since these do not grow on the usual laboratory media their true significance has not been explained.

Summary and Conclusions

Cellulose-digesting organisms, both bacteria and molds, are very abundant in termite nests. These organisms have been obtained in abundance from material dried for a year.

Cellulose-digesting bacteria were not isolated from the intestines of termites on the nitrate-cellulose medium of Bradley and Rettger nor on the silica-gel medium of Winogradsky.

True bacteria are probably of little importance in cellulose digestion in termites.

BIBLIOGRAPHY

Beckwith, T. D., Rose, Edythe J., 1929. Cellulose Digestion by Organisms from the Termite Gut. *Proc. Soc. Exper. Biol. and Med.*, **27**: 4.

Bradley, L. A., Rettger, L. F., 1927. Studies on Aërobic Bacteria Commonly Concerned in the Decomposition of Cellulose. *Jour. Bacteriol.*, **13**: 321.

Buchner, Paul, 1928. Holznahrung und Symbiose. Berlin.

Cleveland, L. R., 1924. The Physiological and Symbiotic Relationships between the Intestinal Protozoa of Termites and their Host, with Special Reference to Reticulitermes flavipes Kollar. *Biol. Bull.*, **46**: 178.

Cleveland, L. R., 1925. The Method by which Trichonympha campanula, a Protozoön in the Intestine of Termites, Ingests Solid Particles of Wood for Food. *Biol. Bull.*, **48**: 282.

Cleveland, L. R., 1925. The Ability of Termites to Live Perhaps Indefinitely on a Diet of Pure Cellulose. *Biol. Bull.*, **48**: 289.

Cleveland, L. R., 1925. The Feeding Habit of Termite Castes and its Relation to their Intestinal Flagellates. *Biol. Bull.*, **48**: 295.

Cleveland, L. R., 1925. The Effects of Oxygenation and Starvation on the Symbiosis between the Termite, Termopsis, and its Intestinal Flagellates. *Biol. Bull.*, **48**: 309.

Cleveland, L. R., 1928. Further Observations and Experiments on the Symbiosis between Termites and their Intestinal Protozoa. *Biol. Bull.*, **54**: 231.

Dore, W. H., Miller, R. C., 1923. The Digestion of Wood by Teredo navalis. *Univ. Calif. Publ. in Zoöl.*, **22**: 383.

Hoelling, B. A., 1910. Die Kernverhältnisse von Fusiformis termitidis. *Arch. Protistenk.*, **19**: 239.

Hollande, A. C., 1922. Les Spirochètes des Termites; processus de division; formation du Schizoplaste. *Arch. de Zool. Expér. et Gén.*, **61**: 23.

Imms, A. D., 1924. A General Textbook of Entomology. London.

McBeth, I. G., 1916. Studies on the Decomposition of Cellulose in Soils. *Soil Science*, **1**: 437.

Waksman, S. A., Carey, C., 1926. The Use of the Silica Gel Plate for Demonstrating the Occurrence and Abundance of Cellulose-Decomposing Bacteria. *Jour. Bacteriol.*, **12**: 87.

Winogradsky, S., 1929. Études sur la microbiologie du sol. *Ann. de l'Institut Pasteur*, **43**: 549.

THE INNERVATION OF THE STOMACH AND RECTUM AND THE ACTION OF ADRENALINE IN ELASMOBRANCH FISHES

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A study of the literature concerning the innervation of the stomach and intestine in mammals reveals much confusion and contradiction. The orthodox differentiation into sympathetic and parasympathetic with antagonistic actions has many exceptions. Thus Langley (1898) found inhibitory fibers to the stomach in the vagus of the rabbit, Morat (1893) found excitatory fibers to the stomach and intestine in the splanchnic of the dog, and Carlson, Boyd and Pearcy (1922) have found that both the splanchnics and the vagi of the cat carry both kinds of fibers to the stomach. On the basis of effects produced by adrenaline, Smith (1918) assumed the splanchnics to be inhibitory for the stomach in man and in the cat, but only for certain parts of the stomach in the guinea pig, rabbit and dog, while being motor for other parts. Tashiro (1920), however, using adrenaline on surviving cat intestine, came to the conclusion that there are motor fibers to the circular muscle in the sympathetic nerves as well as inhibitory fibers to both the circular and the longitudinal layers. McCrea, McSwiney and Stopford (1925) found that in dogs, cats and rabbits the primary effect of stimulation of the peripheral cut end of the vagus on the stomach may be inhibition or augmentation, depending upon the intragastric pressure, but that the final effect is motor. Brown, McSwiney and Wadge (1930) found that the effect of sympathetic stimulation depends on the type of stimulation. A low frequency contracted the body of the stomach in the cat, whereas ordinary tetanizing current inhibited. All rates inhibited the antrum, and adrenaline caused an inhibition of both parts. In a review Van Campenhout (1930) says, "We believe the actual distinction of sympathetic, parasympathetic and local innervations to be erroneous owing to ignorance of the real constitution of the visceral autonomic nervous system." A similar view was expressed by Langfeldt (1929), who concluded that there is no absolute antagonism between the sympathetic and parasympathetic and that our information concerning the peripheral termination of both systems is incomplete.

The literature concerning the visceral innervation in the lower vertebrates shows no more conformity to the orthodox view than does that in mammals. Goltz (1872) showed that the splanchnic nerves are motor for the stomach in the frog, and this has been confirmed by Dixon (1902), Müller and Liljestrand (1918) and Itagaki (1930). Dixon (1902) found the vagus in the frog to have either an inhibitory or a motor effect on the stomach. For reptiles there is not enough evidence to make a comparison, either anatomical or physiological, although Thorell (1927) by the use of adrenaline considered the sympathetic to be inhibitory to all parts of the turtle's stomach except the cardiac portion. In birds Nolf (1925) has reported that the vagus is motor to the crop, and either motor or inhibitory to the gizzard and small intestine; and the coeliac nerves are either motor or inhibitory to the gizzard and duodenum.

In elasmobranch fishes the autonomic nervous system appears not to be well developed (Müller and Liljestrand, 1918). Bottazzi (1902) found both the vagus and the anterior splanchnic nerves in *Scyllium canicula* to be motor for the stomach. He was unable to demonstrate any inhibitory effect of either. Stimulation of the cord in the region from the forty-fifth to forty-eighth spinal nerves gave motor activity of the rectum. Müller and Liljestrand (1918) confirmed Botazzi (1902) in part, using *Squalus acanthias* and various species of *Raia*, but believed an inhibitory effect of the vagus on the stomach to be more marked than the motor effect. They never obtained evidence of inhibition from the anterior splanchnics. Stimulation of the middle and posterior splanchnic nerves was without effect on the spiral valve and rectum.

In view of other peculiarities of the autonomic nervous system in elasmobranchs, namely, the lack of accelerator nerves to the heart (Bottazzi, 1902; Müller and Liljestrand, 1918; Lutz, 1930*a*) and the inhibitory action of adrenaline on the heart (Macdonald, 1925; Lutz, 1930*b*), the present writer believed that it might prove useful to compare the effects of adrenaline and extract of chromaphil tissue on parts of the gut with the effects of electrical stimulation of the extrinsic nerves to the same parts.

MATERIAL AND METHOD

The elasmobranchs used were *Squalus acanthias*, *Raia erinacea* and *R. diaphanes*. For anatomical reasons only *S. acanthias* was used when nerves were stimulated. Segments of the stomach, spiral valve, and rectum one half to one inch long were suspended in 50 cc. of a physiological solution described by Lutz (1930*b*), and tracings obtained. Some pieces were hung so that the circular muscles would activate the lever, others were suspended so that the longitudinal layer would be most effective. Adrenalin chloride (Parke, Davis & Co.) and chromaphil tissue extracted in distilled water were added to the bath by means of a pipette. Control tests, in which similar amounts of distilled water and extracts of liver and spleen were added to the bath, showed that the method was satisfactory. An extract of the anterior chromaphil bodies was made in one cc. of distilled water immediately on removal of the tissue, and used at once. In a few cases, in which *R. stabuliforis* served as a source of chromaphil tissue, only one "axillary heart" was used to one cc. of distilled water, but in the case of the smaller species of *Raia* both anterior chromaphil bodies were used and sometimes, in addition, some of the accessory bodies.

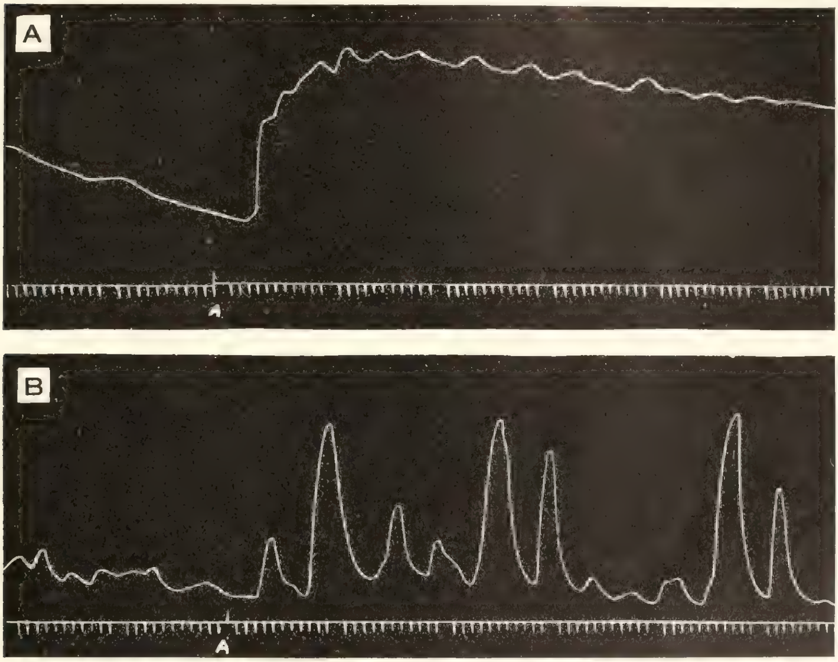

FIG. 1. Effect of adrenalin chloride, 1 in 50,000, on the pyloric portion of the stomach. Time in 5 second intervals. *A*, *Raia erinacea*. Typical effect on tonus. *B*, *R. diaphanes*. Effect mainly on motility.

In the experiments in which the extrinsic nerves were stimulated the entire central nervous system was pithed. The left vagus was exposed through the anterior cardinal sinus. The first sympathetic ganglion (gastric) and the anterior splanchnic nerves were exposed through the posterior cardinal sinus, or the latter were sometimes stimulated along the course of the coeliac artery. The posterior splanchnic nerves (rectal) were stimulated along the posterior mesenteric artery or in the mesentery supporting the rectal gland. Records of the movement of the gut were obtained by means of a small hook and a light lever. The nerves were stimulated with faradic current by means of platinum electrodes leading from an inductorium (Harvard Apparatus Co.) with the secondary coil set at 8 cm. and one 2.5 volt dry cell in the primary circuit.

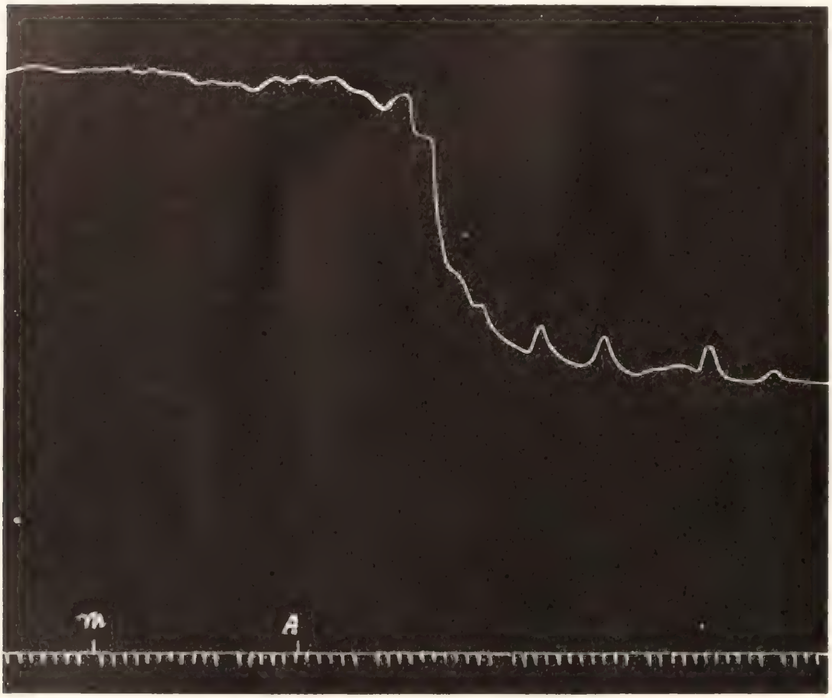

FIG. 2. Effect of adrenalin chloride, 1 in 50,000, on the rectum of *Squalus acanthias*. Time in 5 second intervals. At *M*, 2 cc. of the bath fluid squirted on the preparation. Adrenalin chloride added at *A*.

RESULTS

Adrenalin chloride added to the bath fluid to make one in 50,000 caused a rise in tone and sometimes augmentation of rate and height of the movements of the pylorus and other parts of the stomach in twenty-five preparations and had no effect in three cases (Fig. 1).

In some inactive preparations motility was initiated by a similar dose. A distilled water extract of chromaphil tissue taken from the skate gave the same effect as adrenalin chloride on the pylorus and stomach of both the skate and the dogfish (Fig. 3). Extracts of liver and spleen, agitation of the bath fluid, or the addition of distilled water gave no response.

On twelve preparations of the posterior end of the spiral valve and the rectum, adrenalin chloride, one in 50,000 caused a marked fall in tone and an inhibition of motility (Fig. 2). In no case was there activation or failure of response. Extract of chromaphil tissue also caused inhibition (Fig. 3). Extract of liver gave no response.

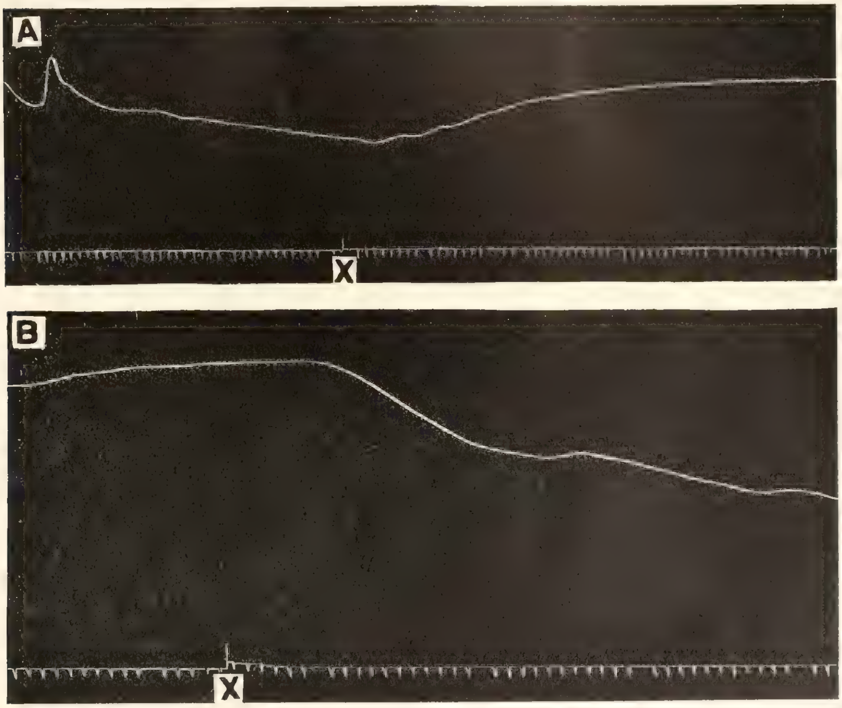

Fig. 3. Effect of extract of chromaphil tissue. Time in 5 second intervals. *A*, *Squalus acanthias*, pyloric portion of the stomach. *X*, extract of the axillary bodies from one side of *Raia stabuliforis*. *B*, *R. erinacea*, rectum. *X*, extract of the axillary bodies of the same specimen.

In thirteen specimens of *S. acanthias* faradic stimulation of the first sympathetic ganglion (gastric) or the anterior splanchnic nerves caused extensive contractions of the stomach beginning in the pyloric region. The latent period varied from two to five seconds (Fig. 4, *A*).

In seven fishes faradic stimulation of the peripheral end of the cut vagus at the anterior cardinal sinus caused moderate contractions of the pylorus and adjacent region. The latent period was about five seconds. In one case no response was obtained. The response from the vagus was never obtained longer than forty minutes after the opening of the cardinal sinuses, whereas the sympathetic response was obtained after three hours.

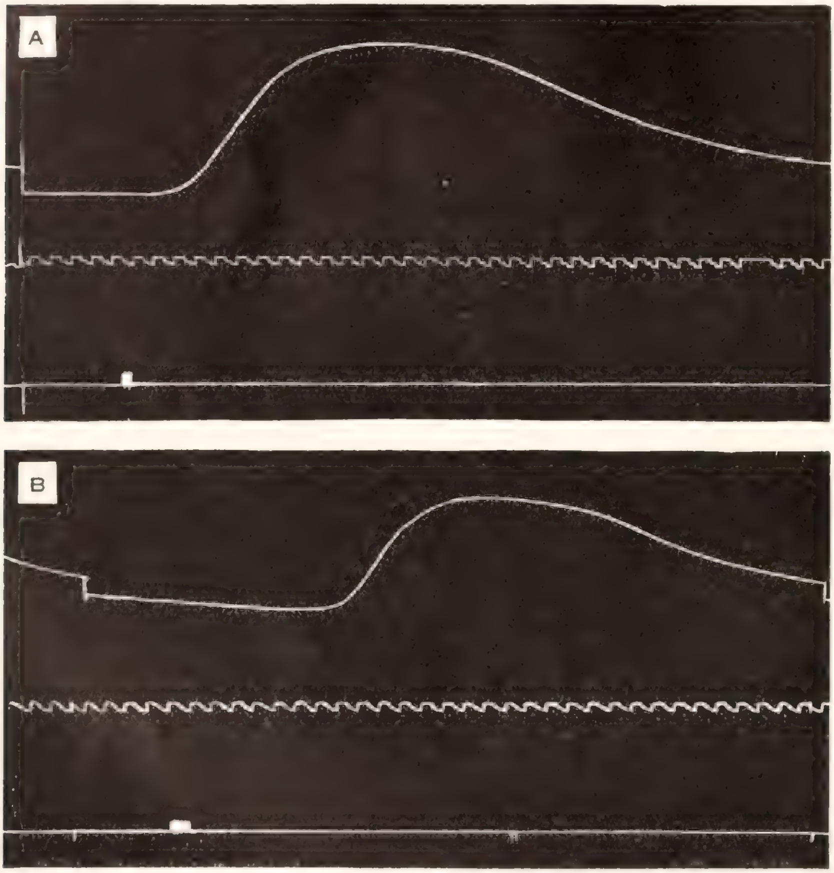

FIG. 4. Effect of faradic stimulation. Time in seconds. *A*, *Squalus acanthias*. Contraction of the pyloric portion of the stomach on stimulating the first sympathetic ganglion (gastric) for one half second. Latent period, 2.5 seconds. *B*, *S. acanthias*. Contraction of the rectum on stimulating the posterior splanchnic nerves for one second. Latent period, 8 seconds.

In four fishes the posterior splanchnic nerves were stimulated and in each case a vigorous contraction of the rectum and the adjacent part of the spiral valve was repeatedly obtained (Fig. 4, *B*). The response had a latent period of eight to ten seconds, and in one specimen was active after three hours without the circulation.

DISCUSSION

The motor effect of electrical stimulation of the sympathetic and of adrenaline on the stomach of the elasmobranch is another exception to the view that in general the sympathetic is inhibitory to the gut and the vagus motor. In this case the effect of adrenaline is sympathico-mimetic. If the posterior splanchnic nerves, stimulation of which activates the rectum, are sympathetic, as Müller and Liljestrand (1918) describe them to be, then the inhibitory action of adrenaline on this part of the intestine is also an exception. Brown, McSwiney and Wadge (1930) found that adrenaline did not reproduce the effects of sympathetic stimulation of the stomach in the cat and in the dog, inhibition being the invariable result.

The results reported here confirm Bottazzi (1902) working on *Scyllium*, and Müller and Liljestrand (1918) working on *Squalus* and *Raia* insofar as the effect on the stomach of electrical stimulation of the anterior splanchnic nerves is concerned. However, since a marked contraction of the rectum resulted from stimulation of the posterior splanchnic nerves, and no evidence of inhibition of the stomach through stimulation of the vagus was obtained, these results are to that extent at variance with those of Müller and Liljestrand.

While there may be a valid reason for perpetuating the morphological division of the autonomic nervous system into cranial, thoracolumbar and sacral parts, there is sufficient evidence to indicate that a general physiological distinction should not be made so far as control of the alimentary tract is concerned.

SUMMARY

1. Adrenalin chloride and extract of the chromaphil bodies caused a rise in tone and sometimes an increase in motility of all parts of the stomach of *Squalus acanthias*, *Raia erinacea* and *R. diaphanes*.

2. Faradic stimulation of the first sympathetic ganglion (gastric) and the anterior splanchnic nerves caused extensive contractions of the stomach beginning at the pylorus in *Squalus acanthias*. Similar stimulation of the vagus caused moderate activity in the region of the pylorus.

3. Adrenalin chloride and extract of chromaphil bodies caused a marked decrease in tone and inhibition of motility of the posterior end of the spiral valve and the rectum in all three elasmobranchs.

4. Faradic stimulation of the posterior splanchnic nerves caused a vigorous contraction of the rectum and adjacent part of the spiral valve in *Squalus acanthias*.

5. The data presented here and the evidence from the literature indicate that a general physiological distinction between the sympathetic and the parasympathetic divisions of the autonomic nervous system should not be made.

BIBLIOGRAPHY

Bottazzi, F., 1902. *Zeitschr. f. Biol.*, **43**: 372.
Brown, G. L., McSwiney, B. A., and Wadge, W. J., 1930. *Jour. Physiol.*, **70**: 253.
Carlson, A. J., Boyd, T. E., and Pearcy, J. F., 1922. *Am. Jour. Physiol.*, **61**: 14.
Dixon, W. E., 1902. *Jour. Physiol.*, **28**: 57.
Goltz, F. 1872. *Pflüger's Arch.*, **6**: 616.
Itagaki, M., 1930. *Jap. Jour. Med. Sci.*, **1**: 105.
Langfelt, G., 1929. En oversikt over den kliniske under søkelse av det viscerale nervensystem og en kritikk de forskjellige prøvers praktiske verdi. Bergen.
Langley, J. N., 1898. *Jour. Physiol.*, **23**: 407.
Lutz, B. R., 1930*a*. *Biol. Bull.*, **59**: 211.
Lutz, B. R. 1930*b*. *Am. Jour. Physiol.*, **94**: 135.
Macdonald, A. D., 1925. *Quart. Jour. Exper. Physiol.*, **15**: 69.
McCrea, E. D., McSwiney, B. A., and Stopford, J. S. B., 1925. *Quart. Jour. Exper. Physiol.*, **15**: 201.
Morat, J. P., 1893. *Arch. de physiol. norm. et path.*, **25**: 142.
Müller, E., and Liljestrand, G., 1918. *Arch. Anat. u. Physiol.*, Anat. Abt., p. 137.
Nolf, P., 1925. *Arch. internat. de physiol.*, **25**: 291.
Smith, M. I., 1918. *Am. Jour. Physiol.*, **46**: 232.
Tashiro, K., 1920. *Tohoku Jour. Exper. Med.*, **1**: 102.
Thorell, G., 1927. *Skand. Arch. f. Physiol.*, **50**: 205.
Van Campenhout, E., 1930. *Quart. Rev. Biol.*, **5**: 217.

LABORATORY REPRODUCTION STUDIES ON THE GROUND SQUIRREL, *CITELLUS TRIDECEMLINEATUS PALLIDUS*, ALLEN[1]

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Introduction

In studies on hibernation carried on in this laboratory for the past six years hundreds of ground squirrels, *Citellus tridecemlineatus*, have been kept in our animal house. During this time many pregnant females have been received in the spring and have usually reared their young, but mating has been known to occur in the laboratory only in two females, both *C. t. pallidus* Allen,[2] the variety used in these experiments. Since the animals were well cared for and were in good health, it seemed desirable to investigate the possible causes of their sterility for the scientific as well as the practical information which such a study might yield. It was expected that reproduction was unlikely to occur except at about the time of the normal breeding season in April and May (Drips, 1919; O. Wade, 1927), but why rut is limited to the spring in this and many other species is another question which studies of the present type may in time help answer.

For the sake of brevity the specific methods and the literature will be considered with the different types of experiments. The majority of the animals in the laboratory served as controls for the special experiments. These controls were kept in wire cages with wood bottoms measuring two by three feet. From one pair to about six animals were usually kept in one cage. Wood shavings were used on the floor and small wooden boxes in which the animals could build nests were usually provided.

Observations on Controls

Since our ultra-violet light, outdoor cage and ovarian extract experiments were performed between March and June, 1930, inclusive, a group of 6 males and 11 females were observed as special controls during these months. In three of these males the testes were partly enlarged and had migrated from the abdomen, the position during the fall and early winter, partly into the scrotum which was enlarging. The latter condition of enlargement of the testes and descent into the scrotum, which becomes darkly pigmented, is typical of the breeding season and animals in this condition will be referred to as "scrotal." Animals showing partial enlargement of the testes and partial descent without enlargement of the scrotum will be called "partly scrotal." In the eleven control females only one had the vagina open between March and June.

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[1] Contribution No. 132 from the Department of Zoölogy, Kansas State Agricultural College, Manhattan.

[2] As these animals were secured in central western Kansas it is possible that some of them may have been *C. t. arenicola* the southern variety recently split off from *C. t. pallidus* by Howell (Proc. Biol. Soc. Wash., 41: 213, 1928).

As the pituitary implantation experiments and one ovarian extract experiment were not limited to the spring, it is important to refer to the sexual development over a longer period than that given for the special controls already mentioned. For years it has been observed that captive male ground squirrels became "scrotal" in late winter and in the spring, some being in this condition when others were not. Several females had also been seen with swollen and even open vaginæ. While the observations from March to June, 1930 showed only a small proportion of animals with enlarged external genitalia, probably because of the lateness, observations made in 1931 with the assistance of Mark A. Foster showed a pronounced development in January and February. In fact a majority of males were "scrotal" January 10. The females, with one exception, showed no external swelling on this date. By February 1 all of the healthy males (26) were scrotal and nine of twelve females more than one year old had very swollen vaginæ, and three of the nine had the vagina open. Of the females which were less than one year old, seven of sixteen had very swollen but not open vaginæ.

Since males and females have nearly always been together in our laboratory there should have been ample opportunity for a great many to breed each year, but none of the animals which have been in the laboratory over winter have yet been known to breed, and only two cases of reproduction among newly received ground squirrels are on record in this laboratory. One female received May 1, 1930 gave birth to a litter on June 22, 1930, and therefore must have bred in the laboratory about May 25. In 1926 one case of breeding in the laboratory had been witnessed on April 24, the day the animals were received. The litter was born about 27.5 days later. This is in agreement with data for *C. t. tridecemlineatus* by Drips (1919), who gives the period of gestation as 28 days, and by O. Wade (1927), who reports two cases of reproduction in the laboratory in which it was between 27 and 28 days.

NUTRITION AND VITAMINS

The animals were fed a ration which we had found to maintain good health and reproduction in rats and mice. It consisted of yellow corn meal (30 per cent), whole wheat flour (30 per cent), skim milk powder (30 per cent), alfalfa meal (4 per cent), bone meal (4 per cent), salt (1 per cent), and cod liver oil (1 per cent). The cod liver oil was added just before feeding. Sprouted oats or green feed and water were also supplied.

The well nourished appearance of the animals showed this diet to be adequate for health. Vitamin A, which may have a slight influence on reproduction (Sure, 1928; Evans, 1928*a*), was present in the cod liver oil, wheat and corn. Vitamin B, whose absence might produce a poor physical state with loss of reproductive power in the male (Evans, 1928*b*; Mattill, 1927) or cessation of œstrus in the female in four weeks, with death some two months later (Parkes, 1928), was supplied by the wheat, corn and alfalfa. Vitamins C and D apparently have little effect on reproduction but they were supplied, C by the green feed and D by the cod liver oil, wheat and green feed. An absence of vitamin E would produce a degeneration of seminal epithelium in the male and an early resorption of the young in the female (Evans, 1925; Evans, Burr and Althausen, 1927), but this vitamin was supplied by the wheat, corn, alfalfa and green feed. The addition of wheat "germ stock" which is especially rich in vitamin E, to the diet of 24 animals through the month of June, which would be the latter part of the normal breeding season, did not affect genital development or reproduction.

PITUITARY IMPLANTS

The work of Smith (1927*a*, *b*) and Smith and Engle (1927) has shown that implantation of the anterior lobe of the pituitary may produce precocious sexual maturity and super-ovulation in mice and rats. These authors have shown that similar results are obtained from implants of the whole gland, indicating that the inclusion of the posterior lobe does not affect the action of the anterior lobe in such implants. In attempting to stimulate the reproductive organs of the ground squirrels, implants of pituitary glands from rats were made into adult ground squirrels. While the entire gland was used, the results obtained should be attributed to the anterior lobe alone as already indicated. About thirty animals were used from November to August. Those used during the summer had been in the laboratory over winter and therefore were at least one year old. A finely cut pituitary from a rat was implanted or injected, with a small amount of physiological salt solution, subcutaneously in the hind leg of the anæsthetized animal by means of a fine glass canula. A striking external genital enlargement was observed in three days, *i.e.*, as a result of two daily implants, in most of the animals. In the female this consisted of a swelling of the vulva and was usually followed by the opening of the vagina after three to six daily implants. In the male the testes usually showed some enlargement and tendency to become scrotal after two implants, and successive implants usually produced still greater enlargement and migration into the scrotum, which usually became more pigmented. The changes in the male were not so pronounced as in the female, for the testes rarely became as large as normal if the implants were made at other times than the breeding season. These genital changes usually persisted about two weeks or more, but they resulted in no cases of reproduction.

Daily implantation of a rat pituitary into each of 8 male and 8 female ground squirrels was begun June 7. By killing one pair on June 8 and each day thereafter a series was obtained, one pair having received one implant each, another pair, two implants, etc., up to a pair which had received eight daily implants each. The ovaries of the females showed a general increase in size, although not showing a perfectly graded series from the one to eight implants, probably because of differences in the animals before the implants were made. A control female, which had not received any implants, and also the experimental female which had received three implants had corpora lutea apparently of œstrus, since no indications of pregnancy were found. This implanted animal therefore did not fit into the series. The ovary of the animal which had received only one implant of one rat pituitary gland contained: numerous small oögonia, without surrounding follicular cells, near the periphery of the ovary; numerous small atretic follicles; and a few moderately large follicles. The animals which had received two and four daily implants showed: an increase in size and number of the larger follicles; some reduction in number of atretic follicles; and possibly some reduction in the number of peripheral oögonia. No corpora lutea were yet present, but a few of the larger follicles in the animal receiving two implants contained blood. The chief change in the animals receiving 5, 7 and 8 daily implants was the tendency of the large follicles to become corpora lutea, and this included even the ones filled with blood. The atretic follicles and also the peripheral oögonia remained about the same in number and size or were possibly reduced in number. Apparently the implants had little effect on the oögonia and the atretic follicles in eight days, but produced a striking increase in size of large or medium sized

PLATE II

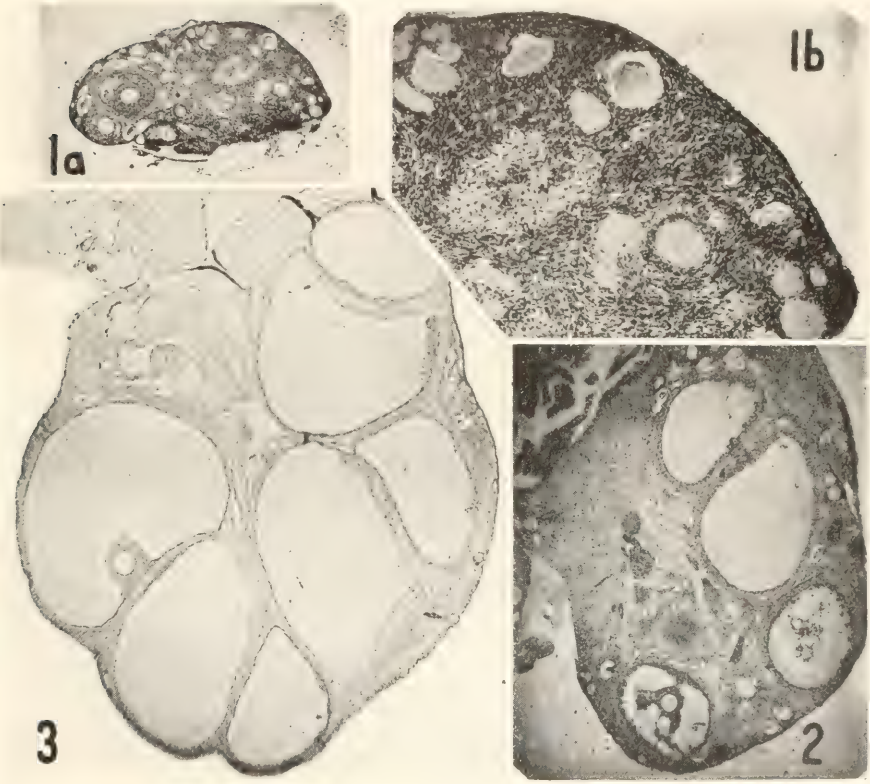


FIG. 1. *a*. A section through a portion of the right ovary of ground squirrel No. 1011 before receiving implants, × 23. *b*. A portion of the same section, × 83. The large follicles measure about 340 microns in diameter.

For further description of the figures see the text.

All the photomicrographs were taken by Mr. Charles Dobrovolny.

FIG. 2. A section through the remainder of the right ovary of ground squirrel No. 1011 after receiving 6 daily implants of rat pituitaries, × 23. The larger follicles measure about one mm. in their greatest diameter.

FIG. 3. A section through the left ovary of ground squirrel No. 1011 after 8 daily implants of rat pituitaries, × 23. The larger follicles now measure about 1.2 to 1.5 mm. in their greatest diameter.

follicles, many of which were hemorrhagic, and finally their development into corpora lutea.

More striking results were obtained from histological study of the ovaries of two females which served as their own controls. On August 8 half of the right ovary of each was removed for study of their normal condition. Each received an implant of a rat pituitary daily from August 13 to 21. The remainder of the right ovary was removed on the seventh day (after 6 pituitary implants) and the left ovary taken on August 22, the ninth day (after 8 daily implants). The normal or control piece of the right ovary of one animal contained a few oögonia without follicles, some atretic follicles and a few young to medium-sized follicles (Fig. 1). After 6 days of implantation the follicles increased in size and some of them were then mature (Fig. 2); after 8 implants these were extremely large, much larger than normal mature follicles, and some contained red blood cells in a part of the follicular cavity (Fig. 3). No marked change in number of atretic follicles and of oögonia could be noted.

The control piece of ovary removed from the other animal, August 8, contained many oögonia without follicles, very many atretic small follicles, some small follicles and some which had grown to the stage of beginning of cavity formation. Six days of implantation produced a few large follicles, most of which contained blood. After eight days the follicles were still larger, some of them containing blood, and corpora lutea were beginning to form, even beginning to hem in the blood in some of the follicles. Two ova were seen in one fallopian tube of this animal. In this and also in the other female killed August 22, one day after each had received eight daily implants of rat pituitaries, the ovaries and uteri were much enlarged and the vaginæ were swollen and open at the time of autopsy.

The males killed, one each day for 8 days, after one to eight days of pituitary implantation beginning June 8 did not show a perfectly graded series as to histological development of the testes, possibly because of variations in condition at the beginning of the experiment. A non-implanted male, killed at this time as a control, showed many more spermatogonia and more spermatogonial divisions than the implanted males, and many primary spermatocyte (spireme) stages, but without more advanced stages of mitosis. The animals implanted one and two days showed little advance over the control. Those implanted three and four days showed testicular enlargement and some descent of the testes into the scrotum, and sections of the testes showed chiefly spermatids and attached spermatozoa in addition to spermatogonia. Metamorphosing spermatids were seen in the four-day implanted animal. In these two males implanted for three and four days, the prostates and Cowper's glands were large, but this was not the case in any of the other 6 males. Probably these two males were more sexually active before the implantations than the others were, otherwise these two should not have been the only ones to show enlargement of these glands. The last four males implanted showed enlargement of the scrotum and some enlargement of the testes but not as marked as in the ones implanted three and four days. A gradual histological change occurred in these four males from a predominance of primary spermatocyte spiremes (after 5 days of implantation) through some stages of active mitosis of the first maturation division (6 and 7 days), to stages of active mitosis of the second maturation division (8 days). Spermatogonia were present, but spermatids and spermatozoa were not found in these four animals.

From each of two males, which had been in the animal house a year, a small piece of testis was removed for study of the testicular condition before pituitary implantation, August 9. These control pieces contained spermatogonia and many primary spermatocyte spireme stages without more advanced mitotic stages (Fig. 4). After four days of healing these two males were given daily implants of rat pituitaries for 8 days and the testes fixed on the ninth day, August 21. The testes of one of the males now contained many spermatids and active division phases of mitosis of maturation (Fig. 5). The testes of the other male now showed a few spermatids and several active division phases of mitosis of maturation (Fig. 6). No metamorphosing spermatids and no spermatozoa were present in either male.

While the development in the gonads was not uniformly progressive in the eight-day series of animals, it is apparent that the implants in the female generally stimulated the growth of the follicles to an unusual size, often accompanied by bleeding into the follicle; and in the male produced a general enlargement of the testes tending towards the formation of spermatids, but without enlargement of the prostate and Cowper's glands to the extent found in breeding males.

Work on the conditions of the reproductive organs at different seasons, and on the effect of pituitary implantation at different times of the year on our laboratory supply of ground squirrels, is being continued by the senior author and Mr. Mark A. Foster.

Ultra-violet Radiations

Saidman (1924) found ultra-violet light beneficial in treating menstrual disturbances. At the suggestion of Dr. R. K. Nabours of this department a mercury arc ultra-violet lamp was placed at a distance of three to four feet above several all-wire cages containing ground squirrels. The lamp transmits slightly more ultra-violet light than is found in sunlight, and will cure rickets in chickens in half-hour daily radiations according to Professor J. S. Hughes of the Chemistry Department of this College. However, radiations of five to twenty-four hours daily had no influence on the genital or general physiological condition of our ground squirrels.

A combination of ultra-violet radiation with pituitary implantation produced no effects other than those of implantation alone.

Ovarian Extract Injections

Œstrus was produced in old albino rats by Slonaker (1927) and in castrated and normal mice by Tuisk (1927) with follicular fluid extracts. Golding and Ramirez (1928), by the use of ovarian and placental extracts, caused the vaginæ of rats to open prematurely with a production of continuous œstrus. Work by Allen and Doisy (1923) and others also show that ovarian extracts may have a marked effect upon genital changes.

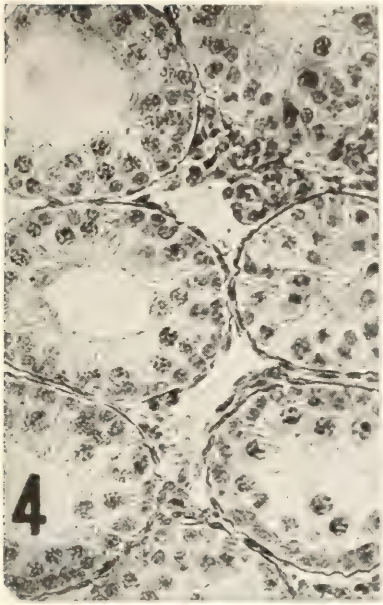
Through the courtesy of the Veterinary Division of this College we procured an alcoholic saline extract of beef ovaries from which the corpora lutea had been removed. This extract had been used with success upon non-producing cows by McLeod (1929) and Frank (1929). Injections of one cc. of this extract every third day for the last two weeks in June produced no positive changes in female ground squirrels either with the vagina open or closed, although the dosage was about 200 times as great as that used for cows when relative weights are considered. The females with open vaginæ were placed daily with sexually active males, but no reproduction took place.

Another experiment was begun January 14, 1931. The extract was injected into three female ground squirrels, one cc. daily for 22 days. It produced no apparent effect on the vagina, which was closed and not swollen at the beginning of the experiment. Histological examination of one-third to one-half the right ovary taken from each animal and from one control January 14 showed a large number of young oögonia (primitive ova) peripherially, many young follicles with one row of follicular cells, several small atretic follicles, and a medium number of mature follicles about 300 to 360 microns in their greatest diameter.

Fig. 4. A section through a portion of the right testis of ground squirrel No. 1115 before it received any pituitary implants, × 120.

Fig. 5. A section through the remainder of the right testis of ground squirrel No. 1115 after receiving 8 daily implants of rat pituitaries, × 120.

Fig. 6. A section through a testis of ground squirrel No. 1078 after 8 daily implants of rat pituitaries, × 120.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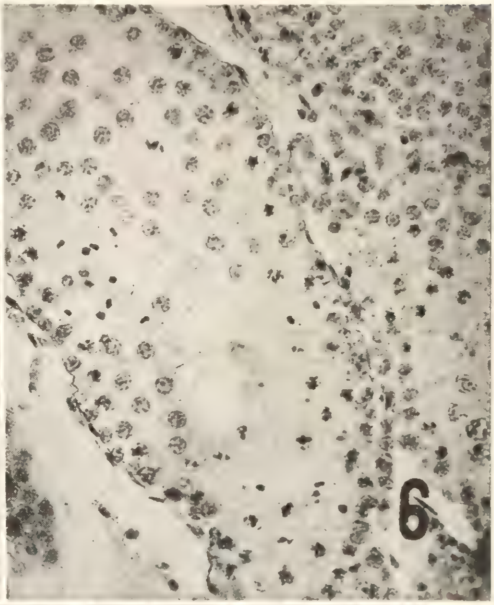
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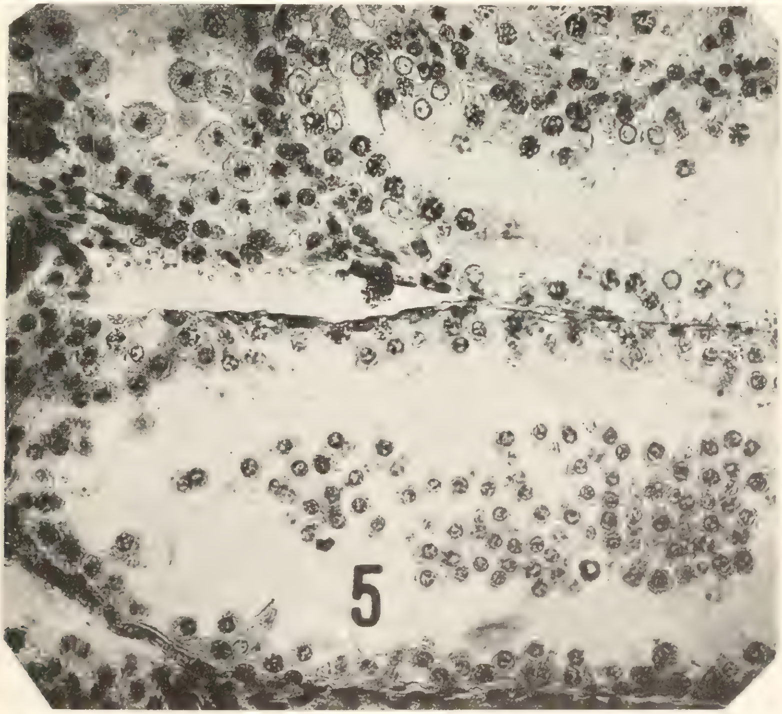
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Careful histological study of the remaining half or two-thirds of the ovary after 13 daily injections showed no marked or consistent change in the two experimentals surviving and in the saline injected control. No histological change was found after 21 days of injection in the one surviving experimental and in the control. In both animals the left ovary was slightly larger than the right had been at the beginning of the experiment, and the uterus had increased about fifty per cent in diameter. Both of these conditions may probably be attributed to the approach of the spring breeding season. The health of these animals was good, the two deaths being produced by the animal chewing into the incision or the somewhat inflamed areas where the injections had been made.

Ovarian Implantation

Ovarian transplantation in the hands of other workers has had marked effect upon the recipients. Grunert (1927) produced œstrus and pregnancy in cows with homeotransplants. Sippel (1924) reported pregnancy in four women after ovarian grafts, and Tuffier and Bour (1925) reported improved health and return of menses with the possibility of pregnancy after such implants. Pettinari (1925) reactivated an old female dog with ovarian grafts.

These and other reports suggested the possibility that implants of ovarian tissue might stimulate genital functions. It was not expected that the tissue would grow, partly because rat ovaries were used, but that it would release the contained hormone as the tissue was gradually absorbed, as was the case with the pituitary tissue implants. The method and technic were practically identical in the two cases. The animals were implanted every third day for two weeks.

The four females used showed no genital development or changes. These implants were not tolerated as well as the pituitary implants, but no serious ill effects were produced.

Outdoor Cages

Since taking ground squirrels from their native habitat into the laboratory stopped their reproduction, it would seem that they should reproduce if placed in outdoor cages. Six pairs kept in such cages during the month of June showed no genital changes when dug out of their burrows. Whether reproduction would take place if the animals were in outdoor cages in April when most of the mating takes place is not known. Even if they did, this would not show why reproduction did not occur in the laboratory.

Discussion

The Possible Inhibitors of Reproduction in the Laboratory

Since the use of the mercury arc vapor lamp did not aid reproduction, the lack of sunlight would not seem to be an inhibitor of it. The benefits of the outdoor sun should last for some time after the animal is captured. Furthermore, it may be stated that the Columbian ground squirrels mate early in the spring before they have had time to absorb much ultra-violet light (Shaw, 1926). This would be true to some extent for the thirteen-lined ground squirrels, for they have a period of rut of about two weeks, shortly after coming out of hibernation, according to Drips (1919).

As the various vitamins were provided in the diet, lack of reproduction could not be attributed to their absence. The good condition of the animals, the good growth made by young ground squirrels, and also the satisfactory rate of growth and reproduction in the mice and rats fed the same diet give further evidence in that direction.

It cannot be stated that hibernation is not a necessary forerunner of reproduction, but at least it does not greatly aid reproduction in the laboratory, for great numbers of ground squirrels have been received before and during the normal breeding season (April and May), but only two known cases of copulation have occurred after they reached the laboratory, although the females were nearly always left with males. Such animals had, of course, passed through normal hibernation in nature. Ground squirrels which had hibernated in the refrigerator for varying lengths of time never reproduced following their return to the animal house.

While marked development of the reproductive organs was produced in the ground squirrel by the anterior lobe principle in the pituitary implants, this development was not as complete as that obtained by Smith and his associates in the rat and mouse, or by Wolf (1929) in the frog. Ovulation did occur in one of the implanted ground squirrels in August. Possibly the female would have reproduced at this time if the males had not been devoid of spermatozoa. Incidentally, the ovulation in this female suggests that under these conditions at least, ovulation may take place independently of copulation. Drips (1919) stated that ovulation occurs only after coitus as found for the ferret (Marshall, 1922) and rabbit (Hammond and Marshall, 1925). She also stated that corpora lutea persist throughout the summer and prevent the recurrence of œstrus after one litter is born.

Indications were found both in the literature and in our observations that the nervous state of an animal may have a profound influence on reproduction. Borries (1929) reported menstrual irregularities in 27 per cent of 39 college women because of nervous strain. Lafora (1923) considered that sexual frigidity in women was often produced by psychic inhibition. According to Steive (1926, 1927) confinement usually results in sterility and gonadal degeneration in wild animals. Testicular atrophy has been noted in men in prison and war amenorrhea in women has been produced by anxiety and worry. Sterility in women was attributed to nervous strain by Macomber (1924). Dr. Yoder of the State Hospital, Kalamazoo, Michigan, in a letter states that there is a positive correlation between genital functional derangement and insanity.

Observations made on wild rats, captured to supply pituitaries for the implant work, have indicated that nervous conditions produced by confinement tended to inhibit reproduction. These rats would fight so violently that only one or two could be kept safely in a cage. A number of these, where physical injury was eliminated, died from no apparent cause other than nervousness. A few of the tamest of these wild rats were kept for several months in cages, some with wild, and some with white rats, of the opposite sex, but without producing. The two strains will mate, however, for it was found that an escaped albino had mated with a gray male before she was captured again. A gray female, pregnant when caught, gave birth to 6 young but killed them.

The ground squirrels with which we are working never become tame in our laboratory. Gloves are practically a necessity in handling them, since they resist capture and bite freely when cornered. It is interesting to note that those handled most bite the most and those handled least are the least wild. One animal received in the spring was unusually tame but in spite of frequent handling became as wild as the others in a few weeks.

In reviewing our experimental work, we find that all of the methods used to stimulate reproductive activity, including those found valuable in other animals, were largely unsuccessful in the ground squirrels. The ineffectiveness of these methods together with the nervous condition of the animals, taken with the influence of the nervous system on genital function in other animals, suggests strongly that the almost complete failure of the ground squirrels to reproduce in the laboratory can be attributed to a nervous inhibition. If this is true, the nervous condition probably acts indirectly through the endocrine system. It is, furthermore, uncertain if larger doses of the hormones of the proper endocrine organs can override this inhibition. The cause of these abnormal genital conditions may lie in the nervous and endocrine systems working together to interfere with one or more steps in the normal reproductive process.

MARINE BIOLOGICAL LIBRARY MASS.

Summary and Conclusions

1. The ground squirrel, *Citellus tridecemlineatus*, failed to reproduce in nearly all cases under usual laboratory conditions.

2. The diet provided the animals contained all the vitamins which have been found to be necessary for reproduction.

3. Pituitary implants from rats, with or without ultra-violet radiation, did not cause reproduction but did stimulate the uterus and follicles in the ovary to excessive growth and corpus luteum formation. In the male the normal reproductive condition was not obtained at other times than the breeding season, but the implants produced a marked change in the histology of the testes from spireme stages of primary spermatocytes to spermatocyte division stages and spermatids.

4. Ovarian implants, ovarian extract injections, ultra-violet radiations, and keeping animals in outdoor cages caused no apparent effect on the genital functions.

5. The cause of the inhibition of reproduction and of the failure to reactivate the animals by various means may lie in a complex linkage of the endocrine and nervous systems.

LITERATURE CITED

Allen, E., and Doisy, E. A., 1923. An Ovarian Hormone. *Jour. Am. Med. Assn.*, Chicago, **81**: 819.

Bellerby, C. W., 1928. Relation of Anterior Lobe of Pituitary to Reproductive Organs. *Lancet.*, **1**: 1168.

Borries, Kara von, 1929. Zur Frage der biologischen Wirkungen des Frauenstudiums. *Arch. f. Rassen- u. Gesellsch.-Biol.*, **22**: 51.

Drips, Della, 1919. Studies on the Ovary of the Spermophile with Special Reference to the Corpus Luteum. *Am. Jour. Anat.*, **25**: 117.

Evans, H. M., 1925. The Anti-sterility Vitamine Fat Soluble E. *Science*, **61**: 519.

Evans, H. M., 1928*a*. Effects of Inadequate Vitamin A on Sexual Physiology of Females. *Jour. Biol. Chem.*, **77**: 651.

Evans, H. M., 1928*b*. Effect of Inadequate Vitamin B upon Sexual Physiology in the Male. *Jour. Nutrition*, **1**: 1.

Evans, H. M., Burr, G. O., and Althausen, T. L., 1927. The Anti-sterility Vitamine Fat Soluble E. *Mem. Univ. Calif.*, **8**: 1.

Frank, E. R., 1929. Observations on the Use of Ovarian Extract in the Treatment of Sterility in Cattle. *Cornell Veter.*, **19**: 399.

Golding, George T., and Ramirez, F. T., 1928. Ovarian and Placental Hormone Effects in Normal, Immature Albino Rats. *Endocrinology*, **12**: 804.

Grunert, C. H., 1927. Absence of Estrum Corrected by Ovary Transplantation. *Vet. Med.*, **22**: 112.

Hammond, J., and Marshall, F. H. A., 1925. Reproduction in the Rabbit. Edinburgh.

LaFora, G. R., 1923. Sexual Frigidity in Women. *Siglo méd.*, **72**: 1053.

Macomber, Donald, 1924. Prevention of Sterility. *Jour. Am. Med. Assn.*, Chicago, **83**: 678.

Marshall, F. H. A., 1922. The Physiology of Reproduction. London.

McLeod, W. M., 1929. The Use of Ovarian Extracts in Treatment of Sterility. *Cornell Veter.*, **19**: 401.

MATTILL, H. A., 1927. The Relation of Vitamins B and E to Fertility in the Male Rat. *Am. Jour. Physiol.*, **79**: 305.

PARKES, A. S., 1928. The Nature of the Anœstrous Condition Resulting from Vitamin B Deficiency. *Quart. Jour. Exper. Physiol.*, **18**: 397.

PETTINARI, V., 1925. Phenomènes régéneratifs dans les ovaires d'une vielle chienne après greffe ovarienne. *Compt. rend. Soc. de Biol.*, **92**: 1294.

SAIDMAN, J., 1924. Note sur les rayons ultra-violets et le traitement des glandes à sécrétion interne (ovaires). *Bull Acad. de Méd.*, **92**: 938.

SHAW, WM. T., 1926. A Short Season and Its Effect upon the Preparation for Reproduction by the Columbian Ground Squirrel. *Ecology*, **7**: 136.

SIPPEL, P., 1924. Schwangerschaft nach homoioplastischer Ovarientransplantation bei Hypovarismus. *Zentralbl. f. Gynak.*, **48**: 15.

SLONAKER, J. R., 1927. The Effect of the Follicular Hormone on Old Albino Rats. *Am. Jour. Physiol.*, **81**: 325.

SMITH, P. E., 1927*a*. The Induction of Precocious Sexual Maturity by Pituitary Homeotransplants. *Am. Jour. Physiol.*, **80**: 114.

SMITH, P. E., 1927*b*. Genital System Responses to Daily, Pituitary Transplants. *Proc. Soc. Exper. Biol. and Med.*, **24**: 337.

SMITH, P. E., AND ENGLE, E. T., 1927. Induction of Precocious Sexual Maturity in Mouse by Daily Pituitary Homeo and Heterotransplants. *Proc. Soc. Exper. Biol. and Med.*, **24**: 561.

STIEVE, H., 1926. Unfruchtbarkeit als Folge unnatürlicher Lebensweise. Grenzfragen des Nerven und Seelenlebens, **126**: 52. J. Bergmann, Munich.

STIEVE, H., 1927. Die Abhangigkeit der Keimdrusen vom Zustand des Gesamtkörpers und von der Umgebung. *Naturwiss.*, **15**: 951.

SURE, BARNETT, 1928. Dietary Requirements for Fertility and Lactation: The Vitamin A Content of Wheat Oil. *Jour. Agr. Res.* **37**: 93.

TUFFIER, TH., AND BOUR, D., 1925. Menstruation and Pregnancy after Ovary Grafting or Transposition. *Presse méd.*, **33**: 1073.

TUISK, ROBERT, 1927. Protracted Œstrus Induced by Ovarian Extracts. *Jour. Physiol.* **63**: 180.

WADE, OTIS, 1927. Breeding Habits and Early Life History of the Thirteen-Striped Ground Squirrel, *Citellus tridecemlineatus* (Mitchill). *Jour. Mammal.*, **8**: 269.

WOLF, OPAL M., 1929. Effect of Daily Transplants of Anterior Lobe of Pituitary on Reproduction of Frog (*Rana pipiens* Shreber). *Proc. Soc. Exper. Biol. and Med.*, **26**: 692.

A NEW PENTAMEROUS HYDROMEDUSA FROM THE TORTUGAS

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During the month of July, 1929, a radially symmetrical pentamerous leptomedusa was present in very large numbers in the waters off the Dry Tortugas, Florida. This medusa was quite similar to *Pseudoclytia pentata*, described by Dr. A. G. Mayer from the same waters; in fact, it was identical with *P. pentata* in general form, in color, in habits, and in time of appearance. It differed from *P. pentata*, however, in certain important and specific structural characters, to be described below.

Mayer (1910) says of *P. pentata* that it was "exceedingly abundant at Tortugas, Florida, from June to August, 1897 to 1904. In 1905 it was relatively rare . . . and not a single specimen could be found in 1908. . . . In 1909 it again appeared in fair numbers." The animal was thus abundant, in its season, for seven successive years, then rare or not present for four successive years, reappearing in the next year. No extensive recorded towings were made at the Tortugas from 1909 to 1929. Upon the reinvestigation of the pelagic fauna in 1929 (Grave and Burkenroad, 1929), a pentamerous medusa was found to be present during July in such numbers as to be the dominant plankton form, but this medusa was found to be not specifically identical with the form described by Mayer. *Pseudoclytia pentata* was not taken at any time during the Laboratory season, from June 1 to August 18, 1929, while the medusa taken in 1929 had not been found by Mayer in the course of many years of intensive investigation. The fact that so similar a form appeared at the time when *P. pentata* might have been expected to appear suggests the rapid and complete replacement of one species by a related one, perhaps in the usual manner of invasion by a species whose range is thus extended, but perhaps—and this seems a most interesting possibility—through the complete replacement of a parent stock by a successful, recent, and local mutant from that stock. Mayer considered his *Pseudoclytia* as probably derived by mutation from some four-rayed *Clytia*-like ancestor. Both in 1899 and 1909 he found *P. pentata* to be extremely variable, although the variations do not appear to have been in the direction of the medusa which was present in 1929. This last-found form was also extremely variable, and its aberrations appeared to be in the same direction and manner as those of *P. pentata*. The description of the medusa which was present in 1929 follows:

Pseudoclytia longleyi,[1] n. sp.

Adult Medusa.—Bell flatter than a hemisphere, 4 to 8 mm. in diameter. Gelatinous substance of the bell thin and tenuous. Fifteen to 20 simple tentacles with fairly well-developed, roundly conical basal bulbs. Tentacles, when extended, twice the bell radius or more in length; often carried contracted in close helical coils. Numerous shaftless permanently rudimentary tentacle bulbs which vary in degree of development from slight swellings of the ring-canal to bulbs as large and as well-defined as those bearing shafts; from one to five bulbs, usually two or three, between each pair of tentacles. One to three lithocysts, usually one, between each pair of tentacles and tentacle bulbs, so that the usual total number of lithocysts is about fifty. Each lithocyst contains a single spherical concretion. Velum well-developed, wide. There are five straight, narrow radial canals, 72 degrees apart. The five short, small gonads, of oval outline in the male, circular in the female, are situated upon the radial canals at points closer to the bell-margin than to the manubrium. Manubrium flask-shaped. Stomach pentagonal when viewed from the oral or aboral surface of the medusa. Five simple recurved lips, with a

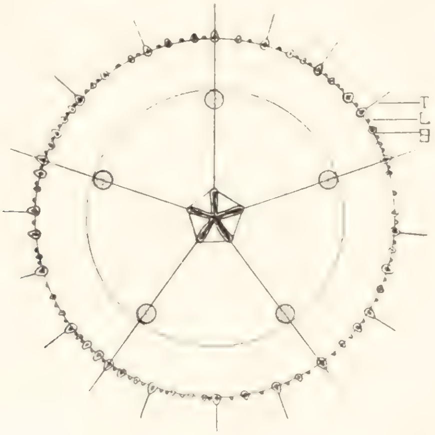

FIG. 1. Pseudoclytia longleyi, n. sp. Diagrammatic. Typical arrangement of the tentacles (*T*), rudimentary bulbs (*B*), and lithocysts (*L*). Tentacle shafts not shown full-length.

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[1] Named for Dr. W. H. Longley.

thin line of nematocysts along their edges. Entoderm of the stomach and gonads, translucently milky. Basal bulbs of the tentacles, and rudimentary bulbs, with a dark entodermal mass. There is an occasional variant, as in *P. pentata*, with brick-red entodermal pigment in the radial canals, tentacle bulbs, and manubrium.

The number and arrangement of the tentacles, rudimentary bulbs, and lithocysts is very variable; even different interradial sectors of the same medusa may differ greatly in this respect. There is often some unevenness in the distribution of the rudimentary bulbs, but without the extreme concentration in one sector described for *Pseudoclytia gardneri* Browne.

Young Medusa.—In medusæ 2 mm. or less in diameter, the gonads are not well-marked. In slightly larger specimens, the gonads are distinguishable as small rounded masses on the radial canals, in about the same position as those of the adult. There were no gonads apparent in the smallest medusa examined, which was .8 mm. in diameter. This medusa had eight tentacles, two tentacle bulbs, and five lithocysts, irregularly arranged, except that there was a tentacle at the end of each radial canal.

The polyp stages of the *Pseudoclytiæ* have not been described. Although some search was made, no hydroids attributable to this species were found. The presence of very early free stages, however, indicates that the fixed stages should be found in the neighborhood of the Tortugas.

The description of *Pseudoclytia longleyi* given above was prepared from living material examined at the Tortugas. The type, and cotypes, are deposited in the United States National Museum, and cotypes are contained in the collection of the Zoölogy Department of Tulane University.

Pseudoclytia longleyi differs from *P. pentata* Mayer (1900) in the following respects:

1. The presence of permanently rudimentary tentacle bulbs.
2. The larger number of lithocysts.
3. The smaller size of the medusa.
4. The situation of the gonads nearer to the bell-margin than to the manubrium.
5. The irregularity of the arrangement of the marginal appendages.

Pseudoclytia longleyi appears to be quite close to *P. gardneri* Browne. This medusa was described (Browne, 1904) from two specimens taken in the Indian Ocean. Mayer (1910) notes that they may have been aberrant specimens of a *Phialidium*. *P. longleyi* differs from *P. gardneri* in the following respects:

1. The larger number of tentacles, rudimentary bulbs, and lithocysts.
2. The symmetrical arrangement of the radial canals.
3. The situation of gonads nearer to the bell-margin than to the manubrium.
4. The greater diffuseness in the distribution of the rudimentary bulbs.

It has been suggested that the medusa described above may be a pentamerous variant, such as has been often noted for many leptomedusae, of *Phialucium carolinæ* Mayer. However, the following facts seem to the writer to strongly indicate that *Pseudoclytia longleyi* must be regarded as a distinct species, and not as a variant of *Phialucium:*

1. *Pseudoclytia longleyi* was present at Tortugas during 1929 in enormous numbers, while not a single specimen of the suggested parent stock, *Phialucium*, was taken. The center of abundance for *Phialucium carolinæ* seems to lie farther north, and it is reported by Mayer (1910) as only occasional at the Tortugas. It is usual, so far as the writer knows, for medusae which differ varietally from a parent stock in the number of radial canals to appear in company with the parent form as aberrant individuals.

2. There are strong structural differences between *Pseudoclytia longleyi* and *Phialucium carolinæ*, aside from the difference in the number of radial canals. Owing to the difficulty of obtaining specimens of *P. carolinæ*, this comparison has been made from the description and figures given by Mayer (1910).

Phialucium carolinæ	*Pseudoclytia longleyi*
Bell almost a hemisphere.	(1) Bell flatter than a hemisphere.
Gelatinous substance of the bell quite thick.	(2) Gelatinous substance of the bell thin and tenuous.
Rudimentary tentacle bulbs much smaller than the shaft-bearing bulbs.	(3) Rudimentary tentacle bulbs often almost as large as the shaft-bearing bulbs.
Lithocysts with two concretions.	(4) Lithocysts with one concretion.
Bell diameter, 14 mm.	(5) Bell diameter, 8 mm. or less.
Entoderm yellow-green.	(6) Entoderm milky or red.
Marginal appendages regularly arranged.	(7) Marginal appendages quite irregularly arranged.

The writer wishes to thank the Carnegie Institution of Washington for the privilege of working at the Tortugas Laboratory.

LITERATURE CITED

BROWNE, P., 1904. Fauna and Geography Maldive and Laccadive Archipelagoes. Vol. 2, Part 3, p. 370.

GRAVE, C., AND BURKENROAD, M. D., 1929. Examination of Pelagic Organisms. *Carnegie Inst. Year Book* No. 28, pp. 283–84.

MAYER, A. G., 1900. *Bull. Mus. Compar. Zoöl., Harvard University*, **37**: 53.

MAYER, A. G., 1910. Medusæ of the World. Vol. 2, pp. 274–76, 278–79.

THE EFFECT OF CERTAIN ENVIRONMENTAL FACTORS ON THE DEVELOPMENT AND HATCHING OF THE EGGS OF BLOOD FLUKES

A. R. ONORATO AND H. W. STUNKARD

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The digenetic trematodes have peculiarly elaborate and complicated life histories. Ordinarily, sexual reproduction in a vertebrate host produces eggs from which a free-swimming larval stage (miracidium) emerges and penetrates into an invertebrate host where repeated asexual multiplication produces a second and different free-swimming larval form (cercaria) which either directly or indirectly, with or without encystment, again infests the specific vertebrate host. Although the general outlines of this complex development have been known for many years and the life cycles of several species have been experimentally demonstrated, the details of the particular processes involved are as yet obscure. The factors operative in the development and hatching of the eggs, penetration of the larvæ into specific hosts, encystment, excystment, and other vital phenomena are almost entirely unknown.

Mattes (1926) studied the development of *Fasciola hepatica*, a species which infests the liver and gall bladder of cattle and sheep, and performed experiments to determine the factors operative in the emergence of the miracidia from the eggs. He found that water at a temperature of 12° C., or above, is necessary for development and that temperatures below − 7° C. will inhibit further development, whereas eggs can recover from exposure to temperatures not lower than − 3° C. He found that the optimum range was between 20° C. and 25° C., at which temperatures the miracidia required from two to three weeks to complete their development. At a range of 14° to 18° C., they required from three to six weeks. Mattes' results indicate also that a sudden drop in temperature may induce the emergence of fully developed larvæ. According to him, a pH of 7.5 to 8 was the most favorable H-ion concentration for development; lowering the pH below 6.5 caused more injury than raising it above 9; and a pH of 5 to 7.5 was the favorable range for hatching. Mattes believed that rain facilitates the emergence of miracidia, since it lowers both the temperature and pH of the water. He found that the miracidia within the eggs are positively phototrophic and that they die if the water becomes putrid or if it entirely evaporates.

Stunkard (1923) described blood flukes from the heart and arteries of various species of turtles and made certain observations on the development of the parasites. *Chrysemys marginata* and *C. picta* harbor species of *Spirorchis*. The flukes deposit their eggs in the blood vessels, by means of which they are distributed throughout the body, especially the visceral organs. The eggs rupture the capillaries and thus gain access to the tissues, through which they work their way to the alimentary tract or one of its derivatives, and ultimately they are voided with the feces. When passed by the turtles the eggs are within mucous pellets which facilitate the collection and manipulation of such minute objects. The eggs of these trematodes appear then to be particularly favorable material for study.

MATERIAL AND METHODS

Since information concerning the factors which influence early development and emergence of trematode larvæ are so important from a purely scientific as well as a medical and economic point of view, it seemed desirable to find out whether Mattes' results with *F. hepatica* are general and whether they are applicable to the eggs of other species. Early in October, 1929, a series of experiments was begun to determine the relation of various factors to the development and hatching of the eggs of blood flukes. The study was made on eggs voided by flukes which infect the turtles named above. Since it is not possible to make specific determination of the eggs, only generic identification is given. These turtles voided many eggs from October until the early part of the following January. For about a month, from the second week of January till the second week of February, the eggs were less numerous, but after this time they appeared again in abundance.

The procedure throughout the study was a comparatively simple one; the turtles were kept in separate aquaria and the eggs were collected daily. The greenish mucous patches, voided by the turtles and in which the eggs are embedded, were picked up in a pipette and placed in small labelled beakers containing tap water which was changed regularly to prevent putrefaction. By this method, a constant supply of eggs was obtained and those containing larvæ of different ages and from different turtles were kept separate.

When the eggs are voided, those in the same mucous pellet vary in their stage of development and in the color of the shell, which is usually a darker brown in the more advanced individuals. This variation has been explained by Stunkard (1923) by the theory that the eggs deposited in various parts of the body of the host require different lengths of time to reach the outside. He also noted that development in the tissues of the turtle is very slow as compared with later development in water, and suggested that the more rapid later development is caused by the absorption of water and by increased oxygen supply. In the present study the development of the larvæ was followed by daily observations on the eggs. It was obviously necessary to wait until the miracidia became mature before experiments could be made to determine the effects of various factors on hatching.

Unless otherwise stated, experiments were carried on at room temperature, since previous work indicated that it is suitable for the development and hatching of these eggs.

The La Motte Standard colorimetric method was used to determine the H-ion concentration of solutions.

OBSERVATIONS

The earlier observations of Stunkard on the contents of the eggs and the course of development were confirmed and extended. A short time after the egg is voided and before the miracidium begins to move, vacuoles can be seen inside the shell. It is probable that these are gas vacuoles and that the gas is carbon dioxide, a product of metabolism of the larva. As development proceeds, the vacuoles become larger due to fusion of the smaller ones and also to the increasing accumulation of gas as the miracidium becomes more active. When the larva is mature there is only one vacuole, often as large as the miracidium itself. This vacuole was shown in Stunkard's figure but was not described by him. The vacuole disappears as the miracidium becomes increasingly active and the question naturally arises as to what has become of the gas and the pressure that must have been caused by it. It seemed possible that the disappearance of the vacuole might be associated with the opening of the shell, but in many instances the vacuole disappeared before the shell gave any evidence of opening. We are not at all certain as to the nature, function, and fate of this vacuole.

In the mature miracidia flame cells were regularly observed; the posterior cells are more readily perceived than the anterior ones. Flame cells have been observed beating continuously for hours, and counts show that they beat approximately 160 times per minute.

The miracidia while still inside the shell are sensitive to light, which is to be expected since they possess well-developed eye spots. The light stimulus usually requires from a few seconds to about three minutes to cause the miracidia to become active. Special precaution was taken to ascertain that it was light and not heat from the lamp that produced the activation of the larvæ. The light was focused on a thermometer placed on the stage of the microscope in the position of the slide and after a 5-minute exposure, the mercury had risen only 0.5° C. Such a slight change in temperature, within the favorable range, could hardly cause the rapid responses manifested by the larvæ when they are stimulated by light.

The shell bears an operculum which is invisible until it starts to open. It is probable that this cap is preformed, since it is always about the same size and at one end of the shell. The removal of the operculum leaves a circular opening through which the miracidium emerges. The escape of the larva was observed many times and the process usually required about five or ten minutes, although occasionally it may take much longer. In one instance a miracidium required 1 hour and 15 minutes to emerge from the shell. The larvæ ordinarily come out anterior end first, but one was observed escaping with its posterior end first. This observation may be significant, as will be pointed out later.

When the cap opens, a fluid mass flows out of the shell along with the larva, and this mass apparently forms an envelope of some kind, for the miracidium has a difficult task to get out of it. Miracidia have been observed for as long as four hours attempting to escape from this viscous fluid. Many of them die after struggling for a long time. It has been observed that the larvæ move about just as freely inside the viscous mass outside the shell as they do inside the shell. It is possible that this substance, which gives a protein reaction, forms a rather flexible membrane when it comes in contact with water. Another possibility, although it is not supported strongly by our observations, is that this is the vitelline membrane of the egg detached from the shell and passed out of the opercular opening with the larva. It is probable that the limiting membrane rather than the viscosity of the fluid prevents the escape of the miracidia since they appear to be continually probing into it. At first the membrane merely gives under pressure and returns to its original position when the pressure is released. If the procedure is repeated often and long enough, the elasticity of the membrane is gradually reduced until it ruptures and the larva is able to escape. It has been noted that if miracidia are liberated into the water as soon as they emerge from the shell they usually disintegrate, and it is probable that during the time they are in the viscous fluid they become adjusted to life in water. If allowed to work their way out of the viscous mass or liberated after some time in it, they swim about so rapidly that it is almost impossible to follow them. They are active for several hours under the conditions of observation.

In three experiments, eggs were placed in a small covered dish containing tap water and fecal material from the turtles. In this putrid water the embryos always died and disintegrated inside the unopened shell. On several occasions the water on the eggs was allowed to evaporate and invariably the shells collapsed and the larvæ died. It is clear that fresh water is necessary at all times.

Although a few miracidia emerged from their shells about three days after their removal from the aquaria in which the turtles were kept, the usual time required for hatching was from 5 to 7 days. Some required longer and a few remained active in their shells for as long as three weeks. Since the eggs were maintained under identical environmental conditions, the variation in time of hatching must be ascribed to other factors. Normal emergence took place at room temperature in ordinary tap water, which was changed every few days to prevent putrefaction. The pH of the water on the fifth, sixth, and seventh days was always between 7.2 and 7.6. It appears that the optimum conditions for hatching are: ordinary tap water, room temperature, and a pH zone between 7.2 and 7.6.

Effects of Acids on Development

Acetic Acid.—To test the effect of acids on development, 74 eggs of *Spirorchis*, voided during the night of November 13th, were placed in a pH 6 solution of acetic acid the following morning. On November 16th one hatched, 43 were moving in their shells, and 30 were still in early stages of development. On November 18th twelve had hatched, 32 were moving in their shells, the rest were still immature. On November 20th sixteen were hatched, 30 were moving, and 28 were in early stages of development. All of the eggs except four eventually matured and by November 26th all had hatched except the four mentioned above. It appears that development was not hastened or retarded at this pH since the time required was practically the same as when the eggs were kept in tap water. It must be recorded, however, that all of the 70 hatched miracidia died either inside the opened shell or immediately in front of the opening, outside of the shell. They did not succeed in freeing themselves from the viscous mass. The results demonstrate clearly that so long as the larvæ remain inside the unopened shell their development is not affected by the acid, but that when the shell opens, they do not long survive.

Lactic Acid.—A similar experiment with lactic acid, pH 6, gave practically identical results.

Effects of Acids upon Hatching

Eggs that had been kept in tap water until the miracidia were mature and moving actively inside their shells were used for these experiments. Since Mattes found that a slightly acid solution induced hatching of the eggs of *F. hepatica*, experiments were made to determine the effects of various concentrations of acetic, lactic, and hydrochloric acids. The first two were selected because they are frequently present at the bottom of ponds in nature.

Acetic Acid.—Mature eggs were placed in a solution, pH 6. Results are shown in Table I, and it is apparent that contact with acid

TABLE I

Effect of H-ion Concentration on Hatching of Eggs Containing Rapidly Moving Miracidia *

Acetic Acid

Time of Observance (*hours*)	pH_6 40 eggs	$pH_{6.5}$ 44 eggs	pH_7* 65 eggs	$pH_{7.5}$* 60 eggs
1/2	None hatched	1 hatched, but larva died almost immediately	None hatched	2 hatched
1	None hatched	Larvæ less active	None hatched	3 more hatched
2	2 shells opened, but larvæ died inside	No more had emerged	None hatched	No more hatched
3	1 hatched, but dead outside shell	No more had emerged	3 hatched	2 more hatched
4	No more hatched	No more had emerged	4 more hatched	No more hatched
6	2 hatched, died outside shell	No more had emerged	3 more hatched	3 more hatched
8	No more hatched	No more had emerged	5 more hatched	4 more hatched
12	Larvæ less active	No more had emerged	8 more hatched	2 more hatched
24	1 hatched, but dead	1 hatched, but died at once	5 more hatched	8 more hatched
30	No more larvæ active	15 hatched, but all were dead	None observed	Some of miracidia were swimming

* It is, of course, understood that there is little or no free acid at pH_7 and $pH_{7.5}$.

at this concentration results in the death of the larvæ. Other eggs were placed in a solution, pH 6.5. The results (Table I) show that this solution is also toxic to hatched miracidia. Tap water was added to some of the above solution to bring it to a pH of 7 and eggs containing mature larvae were placed in this fluid. The results are shown in Table I and indicate that at neutrality the solution is not toxic to the miracidia, since they hatched normally. In order to make a further test, tap water was added to some of the pH 7 solution to raise the pH to 7.5. Eggs placed in this solution also hatched normally.

When eggs were placed in the 6 and 6.5 solutions, the activity of the miracidia was greatly increased. The larvae at first swam about more rapidly and attacked the ends of the shell vigorously. After two or three hours, however, the activity of the larva had decreased to less than usual and in the pH 6 solution, especially, many of them grew weaker until they died. It appears that the acid has at first a stimulating and later a depressing and harmful effect.

Lactic Acid.—A solution of lactic acid with a pH of 6 was prepared and tap water was added to portions of it to make solutions with a pH of 6.5, 7, and 7.5. Mature eggs were placed in each of these solutions and the results are shown in Table II. The effects are not appreciably different from those obtained with similar concentrations of acetic acid. The acid is toxic and the stronger the concentration, the greater the toxicity.

An egg in which the flame cells of the miracidium were beating vigorously was placed in some of the 6.5 solution. At the end of one hour the flame cells were beating much less rapidly and at the end of three hours they were beating very feebly.

Hydrochloric Acid.—A series of flasks was prepared as before. The first contained HCl with a pH of 6, and by adding tap water to portions of this solution others were made up with H-ion concentrations of pH 6.5, 6.8, 7, and 7.5. The effects of these solutions are given in Table III. At a pH of 6, every one of the miracidia died upon coming in contact with the solution. After 24 hours the unhatched larvæ were all dead and after 72 hours they had disintegrated inside the unopened shells. In the pH 6.5 solution the unhatched miracidia were dead at the end of 48 hours. A few experiments, not recorded in the table, were done with HCl at pH 6.8. In one of them, 70 mature eggs were placed in the depression slide. At the end of 24 hours, 34 eggs had hatched. Several of the larvæ had escaped from the viscous masses, although most of them were dead inside the viscous material. In the pH 7 solution the eggs hatched normally

TABLE II

Lactic Acid

Time (*hours*)	pH_6 60 eggs	$pH_{6.5}$ 62 eggs	pH_7 89 eggs	$pH_{7.5}$ 88 eggs
1/2...	None hatched	None hatched	None hatched	None hatched
1.....	1 hatched, but died	None hatched	2 hatched	3 hatched
2.....	4 more hatched, but died	2 hatched	1 more hatched	1 hatched
3.....	No more hatched	No more hatched	2 more hatched	No more hatched
4.....	No more hatched	4 more hatched	4 more hatched	No more hatched
6.....	1 more hatched, but died	6 more hatched	8 more hatched	5 more hatched
8.....	None observed	No more hatched	6 more hatched	2 more hatched
12....	None observed	No more hatched	8 more hatched	3 more hatched
24....	22 had hatched, but all died without swimming	6 more hatched	5 more hatched	8 more hatched
30....		No observation	No observation	At 36 hours 41 eggs had hatched and most of the miracidia had become free-swimming
48....		28 eggs had hatched at this time, but all the miracidia had died inside the shell or near the opening. None had become free-swimming	At this time 66 eggs had hatched and the most of the larvæ had become free-swimming	

and the miracidia were not killed. It appears that this acid at concentrations of pH 6 and 6.5 is lethal to the larvæ and inhibits the hatching of the eggs.

TABLE III

Hydrochloric Acid

Time (*hours*)	pH_6 76 eggs	$pH_{6.5}$ 76 eggs	pH_7 69 eggs	$pH_{7.5}$ 58 eggs
1/2...	None hatched	None hatched	None hatched	In this experiment only 1 egg hatched in 7 days although the larva remained active inside the shells
1.....	2 hatched, but died	None hatched	1 hatched	
2.....	3 more hatched	1 hatched, but died	3 more hatched	
3.....	2 more hatched	None	2 more hatched	No explanation
4.....	3 more hatched	2 more hatched, but died	5 more hatched	
6.....	5 more hatched	4 more hatched, but died	8 more hatched	
8.....	3 more hatched	2 more hatched, but died	3 more hatched	
12....	4 more hatched	3 more hatched, but died	5 more hatched	
24....	8 more hatched rest inactive eggs	3 more hatched, but died	6 more hatched	
48...		At this time 22 eggs had hatched, but larvæ all dead	At end of 48 hours 55 eggs had hatched	

Effects of Various Bases

A series of experiments was also carried out with the bases Na, K, and Ca (Table IV). Solutions were prepared with concentrations of pH 8 and 8.5. The results show that these solutions are all toxic, their toxicity apparently increasing in the following order; KOH, NaOH, and $Ca(OH)_2$.

Effects of Various Temperatures

A number of experiments were made to determine the limits at which these blood fluke larvæ could exist, and also the optimum temperature for development and hatching. If water in which mature eggs were placed was allowed to freeze, the miracidia died although the shells did not collapse. Fifty mature eggs were exposed to a temperature of 0° to 2° C. for 2.5 hours. When removed the larvæ

TABLE IV

Effect of H-ion Concentration and Certain Bases on Hatching of Eggs Containing Rapidly Moving Miracidia

Time (*hours*)	NaOH pH_8 66 eggs	NaOH $pH_{8.5}$ 72 eggs	KOH pH_8 62 eggs	KOH $pH_{8.5}$ 60 eggs	$Ca(OH)_2$ pH_8 85 eggs	$Ca(OH)_2$ $pH_{8.5}$ 74 eggs
1/2...	No effect	No effect	No effect	No effect	No effect	No effect
1.....	No effect	No effect	No effect	No effect	No effect	No effect
2.....	No effect	No effect	No effect	No effect	No effect	No effect
3.....	No effect	No effect	No effect	3 hatched and died, but not as soon as in strong acids	No effect	No effect
4.....	No effect	No effect	2 hatched, but dead	4 more hatched, but dead	No effect	No effect
6.....	No effect	No effect	No effect	No more	2 shells were open, but larvæ were dead in the shell	No effect
8.....	No effect	No effect	3 more hatched, but dead	8 more hatched	All quiet	No effect
12....	No effect	No effect	2 more hatched, but dead	2 more hatched	All quiet	No effect
24....	Few hatched, but dead. Rest dead in the shell	Few hatched, but dead. Rest started to disintegrate	3 more hatched, but dead. Rest dead	9 more hatched, rest dead	All dead	1 open shell, larva dead. Rest all dead in the shell and disintegrated

were quiet, but on allowing the water to reach room temperature again, they recovered and almost all of the eggs hatched during the next 48 hours. Another batch of eggs was subjected to the same temperature for a period of five hours. None of the miracidia recovered from this exposure after being brought back gradually to room temperature. Apparently, a 5-hour exposure at this temperature range is lethal. In the next experiment 30 eggs were placed at 10° C. for eight hours. At the end of that time the miracidia were motionless, but they soon became active when returned to room temperature.

Several eggs were placed in an incubator at 25° C. for 12 hours; during which time about forty per cent of them hatched. A large number of mature eggs were placed at 36° C. for 24 hours. None of them hatched during the time, but sixty per cent of them hatched during the next 24 hours when kept at room temperature. Other eggs were kept at 40° C. for 12 hours and none of the larvæ recovered from the exposure.

Discussion

In the development of the eggs of *Spirorchis* we have described the formation of certain vacuoles which we believe contain a gas, probably carbon dioxide. In many respects these vacuoles resemble the structures described by Barlow (1925) as "oily masses" in the development of the egg of *Fasciolopsis buski*.

There are various theories to account for the opening of the operculum, none of which are entirely free from difficulties and objections. One theory postulates that the activity of the larva is sufficient of itself to force the cap off the shell. Such an explanation was advanced by Johnson (1920) for the hatching of eggs of *Echinostoma revolutum*. It is unlikely, however, that this explanation can apply to the hatching of *Spirorchis* eggs, since miracidia were observed for several days moving actively within eggs without any apparent effect upon the caps.

Another explanation is that the cap is cemented to the main body of the shell and that the cement is gradually dissolved to such a degree that the activity of the larva eventually forces the cap open. Serious objections confront this explanation also. In the course of these experiments mature eggs were kept for many days in water, in acids, and in alkaline solutions without any apparent loosening of the caps. Now if the operculum opens because the cement is dissolved by the surrounding medium, the caps should have come off all of the eggs eventually.

Another theory is that imbibition of water causes a high pressure within the shell, this pressure finally forcing the cap off. One miracidium was seen to emerge with its posterior end first, which is very unusual. By comparing this egg with others in the process of hatching and with hatched miracidia which were re-entering the shell, it was apparent that this miracidium actually emerged with its posterior end first, and that it was not merely a case of a hatched larva re-entering the vacated shell. This observation suggests that internal pressure probably forced the cap off, and simultaneously pushed the miracidium into the opening regardless of its position. The eggs in water tend to become spherical, which indicates absorption of water. Although imbibition may cause an increase of internal pressure, the factors controlling imbibition of water are obscure.

Stunkard (1923) described two cephalic glands in the miracidium of *Spirorchis*, which open near the anterior papilla. Such glands, present in many miracidia, have been regarded by various authors as penetration glands, the secretion of which aids the larva in its entrance into a snail. It is possible that these glands, or others which open slightly behind the anterior end and secrete an oily material, becoming functional when the miracidium is mature, produce a substance which helps to dissolve the cement and loosen the cap. This theory is supported by the fact that the shells do not open until the larvæ are mature, regardless of the medium in which they are placed.

A somewhat similar observation was made by Barlow (1925) on the hatching of the eggs of *F. buski*. In that species he described the formation of a "mucoid plug" during the development of the miracidium and the application of the plug to the inside of the shell in such a manner that it covered the operculum. According to him this mass "seems to serve the purpose of protecting the operculum from the action of the secretions of the miracidium." The process of hatching was described as follows: "This is the evident action of the miracidium as it approaches the mucoid plug. It has a substance to deposit on the plug to erode it and allow of escape. It approaches with caution, applies the erosive to the plug, and then contracts vigorously several times in order to stimulate secretion. When the plug begins to get thin at the apex of the dimple, bubbles begin to show in a little line (Fig. 16) and then these coalesce to form a little tube (Fig. 17). When this tubule finally opens through, the effect on the operculum is instantaneous. No matter whether the miracidium is in contraction or in extension at the time, the operculum flies back on its hinge, water enters the egg, the miracidium becomes violently excited, ciliary motion increases to more than three hundred vibrations a minute, and the miracidium is partly extruded by the hypertonicity of the egg contents and partly assists in its own escape." The anthropomorphic interpretation of Barlow's graphic account appears to lack support, but since conditions in the eggs of *Fasciolopsis* are so different from those of *Spirorchis*, comparisons are not opportune.

The results obtained from a study of the development and hatching of the eggs of blood flukes vary considerably from those secured by Mattes for *F. hepatica*. The miracidia of the latter species require from two to six weeks to develop, whereas those of *Spirorchis* mature and emerge in about a week. The factors which induced hatching of the liver fluke eggs were not effective for those of blood flukes.

Summary and Conclusions

(1) The miracidia are positively phototrophic.

(2) The contents of the egg, which flow out of the shell with the larva, form a membrane which prevents the miracidium from escaping at once into the water. The protein in this substance may be the cause of the membrane formation.

(3) Ordinary tap water is most suitable for the development and hatching of these eggs. Putrid water is toxic to the larvæ.

(4) If the water in the culture is allowed to evaporate, the larvæ die.

(5) The most favorable pH zone for hatching of these eggs lies between 7.2 and 7.6. The miracidia cannot live in a solution having a pH lower than 6.8 or higher than 8.

(6) The optimum temperature for development and hatching is the range 20°–25° C. Exposure to temperature of 0° C. for five hours had a fatal effect upon the miracidia. Although they became quiescent at a temperature of 10° C., they recovered from it on return to room temperature. After extended exposure to temperatures higher than 40° C. the larvæ do not recover.

(7) It is postulated that conditions within the egg shell, rather than those in the surrounding medium, cause the opening of the operculum and emergence of the miracidium.

BIBLIOGRAPHY

Barlow, C. H., 1925. The Life Cycle of the Human Intestinal Fluke, Fasciolopsis buski (Lankester). *Am. Jour. Hyg.*, Monogr. Ser. No. 4.

Johnson, J. C., 1920. The Life Cycle of Echinostoma revolutum (Froelich). *Univ. Calif. Publ. in Zool.*, **19**: 335.

Mattes, Otto, 1926. Zur Biologie der Larvenentwicklung von Fasciola hepatica, besonders über den Einfluss der Wasserstoffionenkonzentration auf das Ausschlüpfen der Miracidien. *Zool. Anzeig.*, **69**: 138.

Stunkard, H. W., 1923. Studies on North American Blood Flukes. *Bull. Am. Mus. Nat. Hist.*, **48**: 165.

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CONTINUOUS VERSUS INTERRUPTED IRRADIATION AND THE RATE OF MUTATION IN DROSOPHILA

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It has been shown by Hanson and Heys (1928) for radium and by Oliver (1930) for X-rays that the rate of mutation in *Drosophila,* as measured by the percentage of sex-linked lethals produced, is determined by the strength of the dose used in the treatments. In general their results justify the conclusion that the rate is directly proportional to the dosage employed. This conclusion is based on results obtained in experiments in which the treatment was given continuously, and in which the only variable factor involved was a difference in the length of exposure. If all other factors are kept constant, the length of the treatment will determine the amount of ionizing radiation to which the flies are exposed. Under these conditions of experimentation they find, for example, that if the time of exposure is doubled, the number of sex-linked lethals produced will also be doubled.

It would seem to be a matter of interest to determine what effect on the mutation rate would follow if the dosage were given in fractions at regularly spaced intervals, instead of continuously as a single dose. Would the summation of the several spaced treatments give a cumulative effect on the rate of mutation, or would the rate be the same as for a single treatment in which the same total dosage was used?

The writer has carried out a series of experiments with a view to finding an answer to this question. In all, 16,963 F_2 cultures have been developed from the several different experiments performed. The method employed was the same as that used by Oliver (1930) and others. The treated flies were tested by Muller's ClB method, which has been fully described by several different writers. The chief point to emphasize is the one already mentioned by Oliver: not all F_2 female cultures are due to point mutation lethals; some result from chromosome abnormalities. Genetic tests were made for the detection of such cases, and those that were found have been excluded from the data listed in the accompanying table. Four separate sets of experiments were carried out, and all of the treated and control P_1 males for a given set were taken from a single culture bottle. The treated flies belonged to a wild-type strain of *Drosophila melanogaster* that had been inbred for many generations, over a period of two years.

TABLE

Groups of P_1 Males	Type of Filter	Target Distance cm.	Nature of Treatment	Time Interval	Total Time	Total Dose in r Units	Number of F_2 Tubes	Lethal Mutations	Percentage of Lethals
1....	alum.	12	continuous		16 min.	1654	971	49	4.95
2....	alum.	12	spaced	12 hr.	16 min.	1654	993	62	6.15
3....	alum.	12	spaced	6 hr.	16 min.	1654	981	71	7.14
4....	card	12	continuous		8 min.	2558	518	39	7.41
5....	card	12	spaced	12 hr.	8 min.	2558	345	45	12.95
6....	controls						986	1	0.10
7....	card	23	continuous		10 min.	1234	863	28	3.11
8....	card	23	spaced	24 hr.	10 min. ±	1220	876	31	3.33
9....	card	23	spaced	12 hr.	10 min. ±	1221	936	40	4.06
10....	card	23	spaced	8 hr.	10 min. ±	1219	856	34	3.76
11....	card	23	spaced	1 hr.	10 min. ±	1220	1014	32	2.94
12....	card	23	spaced	30 min.	10 min. ±	1234	962	33	3.22
13....	alum.	12	continuous		8 min.	864	919	22	2.19
14....	alum.	12	spaced	1 min.	8 min. ±	872	980	21	1.93
15....	controls						951	2	0.21
16....	alum.	3	continuous		12 hr.	radium	544	58	10.44
17....	alum.	3	spaced	12 hr.	12 hr.	radium	452	48	10.40
18....	controls						453	1	0.22

The first series of experiments, which was of a preliminary character, gave results that were not decisive, both because the numbers employed were too small, and because the controls proved to be inadequate. In these tests two groups of larvæ were X-rayed, one continuously and the other intermittently, and two groups of adult males were treated in the same manner. The intermittent exposures were given at regularly spaced intervals, and their sum in each instance was exactly equal to the time of the continuous exposure. The results showed that the groups given the spaced exposures yielded in the F_2 generations a higher percentage of sex-linked lethals than was found in the descendants of flies that had been given continuous treatments. The difference, however, was scarcely statistically significant. It was therefore decided to repeat the experiments on a larger scale, using longer treatments and varying the intervals in the spaced exposures.

In the second set of experiments six different groups of males (all from the same culture bottle) were used. Five of these were treated, and one was used as a control (Table, group 6). The first five groups were treated as follows: Group 1 was exposed for sixteen minutes with the machine operated at 50 kv., peak 10 ma., target distance 12 cm., and a 1-mm. aluminum filter. The total dosage in "r" units (column 7) was calculated from sample readings on a Victoreen dosimeter, taken just after the treatment had been completed. Group 2 was given sixteen exposures, each of exactly one-minute duration, at regular intervals of twelve hours. Group 3 was given thirty-two exposures, each of thirty seconds' duration, at regular intervals of six hours. Group 4 was treated continuously for eight minutes, with the machine operated at the same level as before, but with a card filter of .28 mm. thickness substituted for the aluminum filter. Finally, group 5 was given sixteen exposures, at regular intervals of twelve hours, and each of thirty seconds' duration.

The percentages (corrected for controls) of the tested sex-linked lethal mutations are shown in the last column of the table. It is obvious that groups 2 and 3 should be compared with group 1 and that group 5 should be compared with group 4. Calculations show that the difference between group 1 and group 2 is .012 ± .007. The difference is less than twice its own probable error, and is therefore not significant. If we compare group 3 with group 1, the difference is found to be .0219 ± .0073. Here the difference is exactly three times its own probable error and may or may not be significant. If groups 4 and 5 be compared in a similar manner, the difference is found to be .0554 ± .0138, or slightly more than four times the probable error. It would therefore seem to be statistically significant.

The differences noted above may have been due to chance variations, or they may have resulted from experimental error, incident to starting and stopping the machine. If the sum of the several spaced exposures does not exactly equal (in the total amount of radiation) the time of the continuous exposure, differences in the mutation rate would necessarily occur. In giving the treatments every care was taken to make the two equal. The same voltage, milli-amperage, and target distance were used, and the intervals of time were carefully determined by means of a stopwatch. The total dosages (groups 1 to 5) were calculated, as stated above, from sample readings taken on the dosimeter just after the flies had been exposed, with the machine operated at the same level as was used in giving the treatments. But this method might be a source of error. It was therefore decided to arrange the apparatus so that the dosimeter readings could be taken at the time of making the exposures.

Numerous trial tests showed that so long as the machine was operated at the same level, one obtained very consistent readings.

The next series of experiments (groups 7 to 15) were conducted under the conditions just mentioned. The table shows the various details of the several experiments, except that the length of the individual exposures and the exact manner of calculating the total amounts of radiation as measured in *r* units is not revealed. With reference to the latter point, it may be stated that it was found necessary to vary slightly the length of the last exposure in the series of any given spaced test. This was done in order to make the total number of *r* units correspond as nearly as possible to that used in the continuous exposure. As an example, in group 7, the flies were exposed for ten minutes, and dosimeter readings were taken every thirty seconds. Calculations based on these twenty readings gave a total dosage of 1234 *r* units of radiation. The flies of group 8 were given eight exposures, each of one and a quarter minutes' duration, at regular intervals of twenty-four hours. Two readings were taken during each exposure, and after the first seven treatments had been completed the total dosage up to that point was determined. From the figure obtained, it was found necessary to shorten the last exposure five seconds, in order to treat this group of flies with a total dosage approximating that given to group 7. This gave a total of 1220 *r* units. The succeeding groups were handled in a similar manner. The deviation from the total time set by the continuous treatment was plus or minus about five seconds in each spaced test.

Groups 8 to 12 form a series in which the time interval for the spaced exposures was gradually shortened from twenty-four hours to thirty minutes. The corrected percentages of sex-linked lethal mutations run as follows: continuous (7), 3.11; twenty-four-hour interval (8), 3.33; twelve-hour interval (9), 4.06; eight-hour interval (10), 3.76; one-hour interval (11), 2.94; thirty-minute interval (12), 3.22. From this it will be seen that the percentages vary from 2.94 in group 11 to 4.06 in group 9. If one compares the percentage of lethals for the spaced exposures (groups 8 to 12) with that for the continuous exposure (group 7), one finds that the differences are not significant. The percentage of group 9 shows the widest divergency from that obtained in group 7 (3.11), but calculations show that the difference is .0105 ± .0060, which is less than three times the probable error, and is therefore not statistically significant. Furthermore, the percentage of lethals obtained in the spaced exposures of group 11 is actually lower than that for group 7. Finally, in groups 13 and 14, in which there were eight-minute treatments, the spaced treatment again gave a lower percentage than was produced by the continuous exposure.

From the results obtained in this set of experiments, one may conclude that to give the treatment in fractional doses does not affect the rate of mutation in any way different from that found after a continuous exposure of equal strength had been used. The differences noted are due either to chance variation inherent in the material, or to small uncontrollable fluctuations in the voltage and milli-amperage of the machine. The ideal source of radiation for such a test would have been radium, because the amount of variation in dosage when this substance is used is negligible. Up to the time at which the above experiments were completed (July, 1930) radium was not available, but early last fall, through the kindness of Dr. W. C. Curtis of the National Research Council, 123 mg. of radium were loaned to the laboratory, and this was used in the following test (groups 16 and 17).

The arrangements for giving the radium treatments were such that it was possible to avoid any appreciable error in the time factor. Two groups of P_1 male flies were exposed; group 16 was given a continuous treatment lasting twelve hours, while group 17 was treated with twelve exposures each of exactly one hour, at regular intervals of twelve hours. The F_2 cultures gave for continuous and spaced treatments 10.44 per cent and 10.40 per cent of lethals, respectively. The difference is obviously insignificant.

CONCLUSION

The conclusion that one may draw from these experiments is that the rate of mutation, as measured by the number of sex-linked lethals produced, is the same whether the treatment is given in one dose or in several fractional doses. This is true so long as the total amount of radiation under each of the two conditions is made equal.

The effects of continuous and spaced radiation have been studied by several different investigators, but the only reference found, in which their effects on the rate of mutation are mentioned, is a paper by Serebrovsky and Dubinin (June, 1930), dealing with the production of mutations in *Drosophila* by X-rays. They report (p. 260) that continuous and interrupted exposures were given, and in this connection state that, " It is clear that lengthening the time of exposure with interruption or without raises the percent of mutation." One can not tell from this brief statement whether the percentages were the same or different under the two methods of treatment.

Most of the other papers concerned with methods of administering irradiation doses deal with embryological or histological materials, and they are therefore of no special interest in this connection. One of the latest papers along these lines is by F. G. Spear (May, 1931). Spear used radium and studied and compared the delayed lethal effect on tissue cultures *in vitro,* after spaced and continuous irradiations had been given. He used the subcultivation method and concludes from his results that it is immaterial whether the irradiation is given in one or in several fractional doses—the delayed lethal effect is the same.

Austin, Texas,
June 1, 1931.

REFERENCES

Hanson, F. B., and Florence M. Heys, 1928. The Effect of Radium in Producing Lethal Mutations in Drosophila melanogaster. *Science,* **68**: 115.

Oliver, C. P., 1930. The Effect of Varying the Duration of X-ray Treatment upon the Frequency of Mutation. *Science,* **71**: 44.

Serebrovsky, A. S., and N. P. Dubinin, 1930. X-ray Experiments with Drosophila. *Jour. Hered.,* **21**: 259.

Spear, F. G. 1931. The Delayed Lethal Effect of Radium on Tissue Cultures in vitro—Comparison of Continuous and Spaced Radiation. *Proc. Roy. Soc.,* Ser. B, 108: 190.

GENETIC EVIDENCE FOR DIPLOIDISM OF BIPARENTAL MALES IN HABROBRACON

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I. Introduction

According to the theory of Dzierzon (1845), drones of the honeybee arise from unfertilized eggs. Within recent years this theory has been extended and developed into the general conception that males in Hymenoptera are haploid. It is unnecessary, here, to review the extensive literature dealing with this subject. Despite the evidence for parthenogenetic production of drones, many bee-breeders still insist that certain males in hybrid broods show paternal traits. If Dzierzon's theory is correct, how can this be possible?

The subject of sex determination in Hymenoptera assumes considerable interest to the geneticist from another point of view—that of the theory of genic balance as emphasized by Bridges (1925).

Investigations on the parasitic wasp *Habrobracon juglandis* (Ashmead) have served to clear our conceptions to some extent although much still remains to be done. Significant contributions already published may be briefly mentioned.

It was first shown in 1921 (Whiting, P. W., 1921) that certain males (patroclinous) in *Habrobracon,* contrary to expectation, occasionally show paternal traits and, therefore, do not arise from unfertilized eggs.

Subsequently, Anna R. Whiting found (1925) that such males were often abnormal and almost completely sterile.

The occurrence of new mutations made it possible to show (Whiting, Anna R., 1927) that these irregular males (biparental) resemble their sisters in that they inherit dominant traits from both parents.

It was at first supposed that they might be haploid mosaics. This idea was precluded (Whiting, Anna R., 1928) by the fact that, when the dominant members of two allelomorphic pairs affecting one and the same part of the body (the wings) were contributed by opposite parents, both dominant traits appeared. This indicates, though it does not prove, diploidism.

Still better evidence of diploidism of biparental males appeared (Whiting, Anna R., 1928) when a factor pair was used in which dominance was reversed according to the structures affected. In the series of genes allelomorphic to orange (eye color), "ivory" has eyes and ocelli white while in "light" eyes are black and the ocelli, although of reduced pigmentation as compared with the normal black of wild type, are, nevertheless, dark brown in both males and females of pure "light" stock. Light-ivory compound females have eyes black and ocelli white, thereby exhibiting dominance of "light" in the former, of "ivory" in the latter. Their biparental brothers resemble them in this respect and thus demonstrate a duplex condition of this factor. Such a combination would be impossible in a haploid male.

Another indication of abnormal chromosomal constitution of biparental males is the fact that, occasionally, daughters that are almost completely sterile and often morphologically abnormal are produced (Whiting, Anna R., 1925).

The question as to why biparental males, if diploid, are, nevertheless, males still presents itself. It has been suggested that there may be a sex chromosome for which these males are simplex. The experiments summarized in the present paper were primarily planned for the purpose of locating such a chromosome by genetic test and, in addition, for studying the composition of daughters of biparental males.

The writer is indebted to Professor P. W. Whiting, at whose suggestion the investigation was begun, for guidance as the work progressed. Special thanks are due the co-workers on *Habrobracon* for supplying mutant stocks. Acknowledgment should be made in particular to the Committee on Effects of Radiation on Living Organisms (National Research Council) who, by a grant to Professor Whiting, have furnished technical help which has relieved the writer of much time-consuming labor and thus made possible a greater output of results.

II. Material and Methods

Since the purpose was to locate a sex-linked factor, new mutations were tested with this in mind. In most cases eye color (orange locus) was brought into the cross in such a way, with respect to the mutation, as to serve as an indicator of biparentalism; a male with a dominant eye color being crossed to a female with recessive eye color and the F_1 males with dominant eye color being taken as biparentals. It was not always possible to do this since no light eye colors are present in some of the stocks and, in cases thus far tested, related males and females must be crossed in order to obtain biparental males (Whiting, Anna R., 1925). In these stocks the dominant and recessive allelomorphs of the mutated locus had to be used alone.

The crosses may be divided into five main types involving: 1. normally inherited, recessive mutations in Lancasterized stocks; 2. recessive mutations in No. 11 and Minnesota stocks; 3. female sterile mutations; 4. interacting mutations; and 5. incompletely dominant or incompletely recessive mutations.

The triploid nature of the daughters of biparental males was tested by using some of these same mutations in a manner which will be described later.

III. Presentation of Data

A. Production of Biparental Males

1. Crosses Involving Normally Inherited, Recessive Mutations in Lancasterized Stocks.

Lancasterized stocks have all been graded up to a stock derived from a wild female taken in Lancaster, Pennsylvania in 1919. The method of inheritance by biparental males of certain normally inherited recessive mutations in these stocks has been previously discussed (Whiting, Anna R., 1927). It was shown that, when a female homozygous for the recessive mutant gene was crossed to a male carrying the normal allelomorph, there were produced not only recessive haploid sons but also a few sons with the dominant character. If females, homozygous for the normal allelomorph but marked by some other recessive factor (eye color, usually) were crossed to males, carrying the recessive mutant gene and the dominant marking factor, the biparental males showed only the dominant characters.

A series of quadruple allelomorphs affecting eye color (O, o^l, o and o^i) and three pairs of allelomorphs affecting the wings (W, w), (R, r) and (D, d) were shown to be inherited in the above manner (Whiting, Anna R., 1927).

The fact that of five black-eyed biparental males, obtained from crosses of orange wrinkled (*ooww*) females by type (*OW*) males, one had wrinkled wings, seemed to indicate something unusual about the inheritance of wrinkled wings. As shown in Table I this cross was repeated and 20 biparental males were obtained, none of which had wrinkled wings. The wrinkled wings of the above-mentioned male were no doubt, as they so often are, the result of accident of growth.

As shown further in Table I, three other loci were tested by reciprocal crosses. Long, *l,* affects both the wings and antennæ; eyeless, *el,* and small eye, k^s, or extremely small, k^e, (the latter 3 stocks kindly sent by Dr. Wilhelmina F. Dunning) affect the appearance of the eyes and shape of the head. All three factors were inherited by biparental males in the regular manner described above.

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TABLE I

Crosses Involving Normally Inherited, Recessive Mutations in Lancasterized Stocks

Section	Parents		Matings	Progeny		
	♀	♂		Biparental ♂♂	Impaternate ♂♂	♀♀
1 a	*ooww*	+ (No. 1)	84	19	1219	2220
	ooww	*Y*	11	1	187	322
	ooww	+ (No. 31)	23	0	248	476
	ooww	*l*	11	0	109	208
	ooddww	+	3	5	43	93*
b	*oo*	*w*	50	73	2009	1313*
2 a	$o^io^il(L)l$	*Y*	7	2	87	92
	o^io^ill	*Y*	9	10	42	58
	o^io^ill	+ (No. 31)	2	0	9	11
b	o^io^i (No. 17)	*l*	6	2	68	28
	o^io^i	*l*	20	8	436	479
	cc	*l*	28	18	166	191
3 a	*elel*	+	20	1	203	743
	elel	*wa*	1	6	11	32
b	*oo* (No. 3)	*el*	6	5	164	178
4 a	$o^io^ik^sk^s$	+ (No. 1)	21	41	122	219
	$o^io^ik^sk^s$	*o* (No. 3)	3	3	28	31
b	k^ek^e	k^s	3	3	12	11
Total			308	197	5153	6705

* Taken from Table I—Whiting, Anna R., 1927.

2. Crosses Involving Recessive Mutations in No. 11 and Minnesota Stocks.

An attempt was made to obtain biparental males by crossing tapering, *ta*, (antennæ) males of Minnesota Yellow stock to orange-eyed females of No. 3 Lancaster stock, but none were obtained. Attempts have also been made to cross No. 11 individuals, Iowa City stock, to wasps from Lancaster stocks with the same results. Therefore, in dealing with mutant genes in these stocks, it was necessary to make the crosses between individuals of the same stock and to omit the eye color indicator. It is interesting to note that biparental males do occur when crosses are made within these stocks but do not with outcrossing, a result in agreement with previous findings for other stocks (Whiting, Anna R., 1925).

Table II gives a summary of the data obtained. Inheritance of three mutations: tapering, *ta*, (antennæ—Minnesota Yellow stock); wavy, *wa*, (wings—No. 11 stock) and semilong, *sl*, (antennæ and wings —No. 11 stock) was tested. The data show that wavy females usually produced only unfertilized eggs, though in many cases matings were observed. However, one biparental male was obtained from a wavy female. All three of these mutant genes were found to be inherited in the usual way by biparental males.

Table II

Crosses Involving Recessive Mutations in No. 11 and Minnesota Stocks

Section	Parents		Matings	Progeny		
	♀	♂		Biparental ♂♂	Impaternate ♂♂	♀♀
1	*oo* (No. 3)........	*ta* (Minn.)	10	0	260	431
	tata (Minn.)......	*My* (Minn.)	23	30	608	487
2	*wawa* (No. 11)....	+ (No. 11)	36		383	
	wawa (No. 11)....	+ (No. 11)	2	1	18	2
	elel (?)..........	*wa* (No. 11)	1	6	11	32
3	*slsl* (No. 11)......	+ (No. 11)	17	64	205	184
Total			89	101	1485	1136

3. Crosses Involving Female Sterile Mutations.

Several of the mutant genes in *Habrobracon* seem to have a rather deleterious effect when present in the homozygous condition in the female. In many cases the female has to be given stung caterpillars upon which she is then able to feed and to oviposit, but such females are never as viable as type stock females.

When certain of the genes are present the deleterious effect becomes so great that the females are completely, or almost completely, sterile although the gonapophyses are of normal external appearance.

In dealing with these factors it was necessary to use heterozygous females and to assume that on the basis of chance some of the biparental males would carry the mutated gene. Haploid males with the mutant genes are fertile but usually have more or less difficulty in mating and, therefore, were rather difficult to test.

Table III gives a summary of data obtained from crosses involving four such factors. Fused, *f*, causes fusion of antennal and tarsal segments and an indentation near the apexes of the wings. Miniature, *m*, affects the whole wasps but especially wings and antennæ. Beaded, *b*, causes swelling of the leg segments. Spread, *sp*, causes the wings to be held down and spread out at the sides and produces a light spot on each side of the thorax.

TABLE III

Crosses Involving Female Sterile Mutations

Section	Parents		Matings	Progeny			
	♀	♂		Biparental ♂♂	Impaternate * ♂♂		♀♀
					Dominant	Recessive	
1 a	oo^iFf........	+ (No. 1)	33	33	308	269	451
	oo^iFf........	*f*	15	19	276	277	210†
b	o^io^irr........	*f*	4	8	124		63
	oo (No. 3)...	*f*	1	2	1		6
2 a	o^io^iMm......	*l*	21	8	279	173	503
	Oo^iMm......	*o* (No. 3)	7	0	25	7	144
b	o^io^irr........	*m*	12	0	99		219
	oo..........	*m*	1	2	14		33
	o^io^i........	*om*	3	3	63		32
3 a	o^io^iBb.......	+ (No. 1)	12	6	63	20	181
b	*oo*..........	*b*	14	0	221		229
	oo (No. 3)...	*b*		2			
4 b	*elel*.........	*sp*	4	0	24		132
	oo (No. 3)...	*sp*	10	5	128		247
Total			137	88	1625	746	2450

* Classified as dominant or recessive according to factors *f*, *m*, *b*, *sp* for Sections 1, 2, 3, 4 respectively.

† 114 of these were fused, *ff*.

Biparental males inherited these mutant genes in the usual way.

4. Crosses Involving Interacting Mutations.

Mutations at the three loci (orange, cantaloup and maroon) affecting eye color are, as one would expect, complementary to one another in the effect which they have upon eye color.

Crosses were made (Table IV) in such ways that the dominant of one locus was brought in with the recessive of another, by one parent while the allelomorphs were brought in by the other parent. For example, ivory females (o^io^iCC) were crossed with cantaloup (Oc) males. In addition to ivory-eyed males and black-eyed females, 25 black-eyed males were obtained. These biparental males, like their sisters, received the dominant allelomorph to cantaloup from their mother, the dominant allelomorph to ivory from their father. These complementary dominant factors produced black eye color.

TABLE IV

Crosses Involving Interacting Mutations

Section	Parents		Matings	Progeny		
	♀	♂		Biparental ♂♂	Impaternate ♂♂	♀♀
1 a	*cc*	*o*	53	1	273	698
	cc	+	28	18	166	191
b	o^io^i	*c*	10	25	106	73
	oo	*c*	35	17	305	399
2 a	*mama*	*o*	9	11	52	140
	mama	+ (No. 1)	8	2	35	53
b	*oo* (No. 3)	*ma*	20	36	431	340
Total			163	110	1368	1894

Reciprocal crosses involving the two loci orange and cantaloup and other crosses involving the two loci orange and maroon, were made. In all cases biparental males showed, by having type (black) eyes, that both dominant factors were present in their chromosomes.

5. Crosses Involving Incompletely Dominant or Incompletely Recessive Mutations.

An example of a factor pair with dominance reversed according to structure affected was cited in the introduction. Biparental males were found (Whiting, Anna R., 1928) to resemble their sisters in showing dominance of " ivory " in ocelli but of " light " in eyes, thereby demonstrating the presence of the two allelomorphs " light " (ocelli) and " ivory " (eyes) at the orange locus.

Inheritance of four other genes: dahlia, o^d, (eyes); stumpy, *st*, (legs); yellow, Y, (antennæ) and short, sh, (wings) which produce distinct heterozygous types, has been studied (Table V).

Dahlia, o^d, is one of the quintuple allelomorphs (O, o^l, o^d, o, o^i) of the orange locus. It was noted that dahlia-orange (o^d-o) and, more especially, dahlia-ivory (o^d-o^i) compound females were much lighter than homozygous dahlia females.

TABLE V

Crosses Involving Incompletely Dominant or Incompletely Recessive Mutations

Section	Parents		Matings	Progeny		
	♀	♂		Biparental ♂♂	Impaternate ♂♂	♀♀
1	oo (No. 3)........ o^io^i	o^d o^d	17 37	10 69	112 1080	118 741
2	o^io^istst.......... o^io^istst	o (No. 3) + (No. 1)	10 7	2 0	27 21	45 39
3 a	oo^iYY.......... $oo^i/o\ YY$......	o (No. 3) + (No. 1)	10 41	0 93	38 599	89 833
b	oo (No. 3)...... o^io^i (No. 17).....	Y Y	13 84	1 70	199 801	319 950
4 a	$ooshsh$ .... $ooshsh$..........	+ (No. 1) + (No. 11)	171 4	81 0	3127 74	3572 42
b (1) (2) (3)	o^io^i (No. 17) ..... o^io^i (No. 17) ..... o^io^i (No. 17).....	osh osh osh	26 32 40	7 0 52	360 332 730	321 271 587
Totals			492	385	7491	7927

Crosses such that dahlia-ivory (o^d-o^i) and dahlia-orange (o^d-o) compound biparental males would be obtained, if possible, were made (Table V). Seventy-nine males were found which closely resembled their sisters, the compound (o^d-o and o^d-o^i) females, and were strikingly different from ivory or dahlia or orange haploid males. It seems fair to assume, since they also bred like biparental males, that these males must have been heterozygous and, therefore, duplex for eye color.

Eye color was always used as an indicator of male biparentalism in dealing with the other genes which affect variable traits.

The mutant gene stumpy, st, (legs) is an incomplete recessive. Stumpy wasps have extreme crowding and irregularity of tarsal segments so that, unless close examination is made, the legs appear to end with the tibiæ. Heterozygous females show tarsal segments of approximately normal length but irregularly set together. The majority of these females, moreover, possess a new structure (an added spur on the prothoracic metatarsi resembling the prothoracic tibial spur) never found on type wasps.

Two biparental males were obtained (Table V) from crosses of stumpy females by type males. Both these males had the metatarsal spurs on both prothoracic legs and must, therefore, have been heterozygous for stumpy.

The factor yellow, *Y*, (antennæ) when wasps are reared under standard conditions (30° C.), changes the color of the three basal segments of the antennæ from the normal black to a clear yellow. The character is dependent for its typical expression upon this constant high temperature.

Reciprocal crosses were made (Table V). Wasps of those counts, only, in which biparental males appeared were preserved in alcohol as it was considered that these were reared under the most similar conditions. Later the preserved wasps were graded according to the scheme (devised by Lysbeth Hamilton Benkert) shown in Table VI.

Table VI

Frequency Distribution of Wasps of Various Genetic Compositions According to Grade of Yellow (Antennae)

Experiment No.	Genetic Composition	Class Values *							Mean Value
		1	1.5	2	2.5	3	4	5	
I	Oo^iYy ♀	42		36		59	16	18	2.6
	Oo^iYy ♂	17		15		17			2.0
	o^iy ♂	120		40		1(?)			1.2
II	$Oo^i(o)Yy$ ♀			64	85	21			2.4
	$Oo^i(o)Yy$ ♂	4	23	50		5			1.9
	oY ♂			9		24			2.7
	oY ♂			32		62			2.7

Scheme of grading devised by Lysbeth Hamilton Benkert.

* 1—joints all dark.
2—3 joints yellow, slightly sooty.
3—3 joints clear yellow.
4—3½ joints clear yellow.
5—4 joints clear yellow.

The data show clearly that the biparental males are intermediate between the two types of haploid males (type and mutant) with respect to this character. The biparental males also differ somewhat from their heterozygous sisters. Since females are normally lighter than males of the same stock this difference between heterozygous females and heterozygous males may be explained as a secondary sexual trait.

Variation in the character short, *sh,* (wings) depends upon multiple factors but there is, evidently, one main genic difference distinguishing short stock from type. Short overlaps with normal, especially at lower temperatures. Higher temperatures during development increase the difference from type.

In Experiment I orange short (No. 28) females were crossed to type (No. 1) males and black-eyed males were selected as biparentals. To make it possible to recognize biparental males on the basis of eye color in the reciprocal cross, it was necessary to use an ivory (recessive to orange) stock (No. 17) instead of the black (dominant to orange) stock (No. 1).

Since short is such a variable character, it was considered necessary to make measurements of the wings and to treat the data statistically. When biparental males were obtained, these and equal numbers of brothers and sisters from the same count were preserved in 95 per cent alcohol. Later the right mesothoracic wing and the head of each wasp were removed and placed on a slide. The parts were covered with separate pieces of cover glass and kept moistened with alcohol. Camera lucida measurements were made (magnification 50 ×) of the costal margin of the wing from the tip of the tegula to the end of the radius (point of fusion of R_1 and R_3). This may be called costal length, *c.l.* Similar measurements were taken of head width at the widest point including the eyes, *h.w.* The mean ratio of costal length to head width, *c.l./h.w.,* was used as a basis for comparing the various types. Table VII presents a list of the calculated means and mean differences.

Data given under Experiment I (Table VII) have been previously discussed (Torvik, Magnhild M., 1929). The black males, though they came from mothers homozygous for short, showed a significantly (4.23 × S.E.) greater mean *c.l./h.w.* ratio than that of their short brothers, demonstrating the presence of the type gene (*Sh*) in their chromosomes. Wings of females average somewhat larger than wings of males of the same stock. The mean *c.l./h.w.* ratio of the heterozygous females (*Sh sh*) was greater (6.34 × S.E.) than that of their biparental brothers, showing that these males must also be carrying short (*sh*). They must, therefore, be duplex for this factor (*Sh sh*).

Lower mean *c.l./h.w.* ratios were obtained for both biparental males and heterozygous females of Experiment II than for those of Experiment I. This difference indicates that short is a multiple factor character and that stocks No. 1 and No. 17 differ with respect to some of the factors.

In Experiment II wasps were first reared at the temperature of the former experiment (26°–27° C.) (Table V, line 1). Later an attempt was made to start the young at this temperature and then to shift the larvæ to another incubator running at a higher temperature (35° C.). As seen in Table V, line (2), from 32 crosses no biparental males were obtained and many dead larvæ were observed in vials thus transferred.

The cross was repeated at Woods Hole during the summer of 1929 and wasps which were later measured were obtained (Table V, line 3). The incubator at Woods Hole was kept at 30° C. rather than 26°–27° C. Also, these wasps were reared during the summer, when taking the vials from the incubator to transfer the mothers would have a less cooling effect upon the larvæ than the same treatment of larvæ reared during the winter. These facts help to explain the differences in mean ratios of wasps of Experiment I and Experiment II.

Table VII

Mean Ratios, Costal Length to Head Width, for Wasps of Various Genetic Compositions

Exp. No.	Genetic Composition	Group	$\frac{c.l.}{h.w.} \pm S.E.$	Group Differences	
I	*OoShsh* ♀	*a*	3.551 ± 0.025	*a–b*	0.215 ± 0.030
	OoShsh ♂	*b*	3.336 ± 0.023	*b–c*	0.121 ± 0.028
	osh ♂	*c*	3.215 ± 0.017		
II	*ooshsh* ♀	*d*	3.344 ± 0.019	*d–g*	0.158 ± 0.026
	o^io^iShSh ♀	*e*	3.590 ± 0.020	*e–g*	0.404 ± 0.028
	oo^iShsh ♀	*f*	3.439 ± 0.014	*f–g*	0.253 ± 0.023
	oo^iShsh ♂	*g*	3.186 ± 0.018	*h–g*	0.259 ± 0.023
	o^iSh ♂	*h*	3.445 ± 0.014	*f–d*	0.095 ± 0.024
				e–f	0.151 ± 0.024
III*	*OSh* (No. 1)		3.566 ± 0.018	*h–f*	0.006 ± 0.019
	osh		3.182 ± 0.018	*e–d*	0.246 ± 0.026
				e–h	0.145 ± 0.024
				c–g	0.029 ± 0.025
				h–b	0.109 ± 0.027

* Data collected by Dorothy A. Binns.

In addition to the measurements of the three sorts of offspring—ivory males, orange males and orange females—of Experiment II, homozygous orange short (No. 28) females and ivory (No. 17) females were also measured. An interesting fact was brought out by these measurements (Table VII), namely, that the *c.l./h.w.* ratio is always greater for the female than for the same type of male. Heads of stock No. 17 males and females were approximately the same average width (male head 0.014 cm. narrower by camera lucida measure at × 50), but the male wing was shorter than the female wing (0.352 cm.). For this reason the male ratio of *c.l./h.w.* would be proportionately lower than that of the female.

This point must be borne in mind in interpreting the data. It accounts for the fact that there is no significant difference between the mean *c.l./h.w.* ratio of heterozygous females and of No. 17 (type wings) males while the mean *c.l./h.w.* ratio of the heterozygous, biparental males is very much lower than either. The latter ratio is lower than the mean *c.l./h.w.* ratio of the heterozygous females, presumably of the same composition, because comparison is being made between males and females. It is lower than that of the No. 17 males because they are carrying only the gene for normal wings, while the biparental males show in this way that they are also carrying the gene for short wings.

The data also indicate (Table VII) that short is more dominant than recessive. The heterozygous females are more like the short females than like the No. 17 females (mean differences 4 × S.E. and 6 × S.E.).

It was thought that the mean *c.l./h.w.* ratio for short males from experiment I data supplemented by the same ratio for short females from stocks used for experiment II could be used in making comparisons with that ratio for biparental males of experiment II. Since the small change in temperature appears to have had an unexpectedly pronounced effect, and since male and female ratios are so different, these comparisons can not well be made. The biparental males of Experiment II are, however, very significantly different from their haploid (type wings) brothers with respect to mean *c.l./h.w.* ratio (Difference —11 × S.E.). It is, thereby, clearly demonstrated that short was inherited from the father by these biparental males.

Data supplementary to that of Experiment I were obtained from Dorothy A. Binns (Table VII, Ib). The wasps measured by her were reared under the same conditions as those of Experiment I.

The conclusion that biparental males are heterozygous for short seems justified for three reasons. 1. In Experiment I biparental males were shown to inherit short from their mother and in Experiment II they were definitely shown to inherit it from their father. 2. The mean *c.l./h.w.* ratio for biparental males of Experiment I is significantly different (4 × S.E.) from that of their short brothers and is also significantly different (4 × S.E.) from that of the type (wings) males (No. 17) of Experiment II. The difference from that of the type males (No. 1) of Experiment I would be even greater since No. 17 probably carries minor factors for short. 3. On the basis of supplementary data (Table VII, Ib) biparental males of Experiment I are shown to be intermediate between and markedly different from the two types of haploid males (normal and mutant) in mean *c.l./h.w.* ratio.

It may then be concluded from this study of inheritance of four factors determining distinguishable heterozygous types that biparental males are duplex for these factors.

B. Tests of Biparental Males

One of the strongest proofs of the diploidy of biparental males is the manner in which they breed. It was previously shown (Whiting, Anna R., 1927 and 1928) that over seventy-five per cent of biparental males were entirely sterile and that the remainder had produced a very small number of daughters which showed, with but rare exceptions, the dominant traits of the male parent. A few exceptional cases were reported early in the work on *Habrobracon* (Whiting, P. W., 1921). Seven biparental males with black eyes produced daughters with orange eyes; these males, therefore, bred like mosaics. Another exceptional male produced a fertile daughter with recessive eye color (Whiting, Anna R., 1927). As suggested, this may have been a thelytokous daughter of the female used for the test. One exceptional male was found (Whiting, Anna R., 1927) to breed as a heterozygote.

Table VIII gives a summary of the tests of biparental males made in connection with this study. Whenever possible, males from each type of cross were tested by mating them to females homozygous for the recessive factor involved. Individual males were sometimes mated to different females on successive occasions. Since this was in part a test of sterility, matings were always observed.

The data show that 90 per cent of the biparental males tested were sterile. Occasionally, one of these males had abnormal abdominal sclerites, abnormal legs, wings or antennæ but most of them appeared to be normal and all of them mated with females. There must, therefore, have been something abnormal about the spermatozoa or spermatogenesis of these males.

On the basis of tests here reported (Table VIII) we would expect one out of ten biparental males to produce daughters. Only one daughter was obtained from 90 matings for which 34 biparental (*Sh sh*) males were used (Table VIII). Sixteen daughters were produced by 5 of the 12 biparental (*Ta ta*) males tested. Only 17 matings of *Ta ta* males were observed and of these six were fertile. Four biparental *Sl sl* males, out of 20 tested, were fertile. Fertility of biparental males seems to differ with the factor involved and perhaps with the stock, though this point has not been tested particularly and is merely indicated by the above figures.

TABLE VIII

Tests of Biparental Males

Composition of Males	No. of Males Tested		No. of Matings		Offspring	
	Total	Fertile	Total	Fertile	♀♀	♂♂
WwOo	17	2	33	2	2	862
LlOo	9	1	9	1	1	153
Elel	5	0	5	0	0	503
$K(k^s)k^sO(o)o$ *	14	3	16	3	18	1189
Tata	12	5	17	6	16	1184
Wawa	3	0	3	0	0	173
Slsl	20	4	35	4	25	1264
$FfO(o)o^i$ *	12	0	17	0	0	687
MmOo	2	0	2	0	0	47
BbOo	2	0	3	0	0	134
SpspOo	1	0	1	0	0	2
CcOo(O) *	22	4	29	5	17	904
MamaOo(O) *	16	1	28	1	1	373
$o^d o^i$	14	3	16	3	5	677
$Ststoo^i$	2	0	5	0	0	517
$YyO(o)o^i$ *	38	2	56	2	5	2586
$ShshO(o^i)o$ *	34	1	90	1	1	2685
Totals	223	26	365	28	91	13934

* Symbols set in parentheses may be substituted for immediately preceding symbols.

C. Genetic Composition of Daughters of Biparental Males

Daughters of biparental males were found (Whiting, Anna R., 1925 and 1927) in previous experiments to be more nearly sterile and more abnormal than the biparental males. Certain of the 91 females obtained during the course of this investigation were also somewhat abnormal. Some of them had wrinkled wings or abnormal antennæ or irregular abdominal sclerites and a few had irregular gonapophyses, but only 7 were not tested with caterpillars. Seven died without stinging the caterpillars. Stung caterpillars were used with most of the others. Of the 77 thus tested 66 fed on the caterpillars but laid no eggs, 7 laid eggs which dried up without developing and 3 produced offspring.

The 3 fertile daughters, type in appearance, all came from one of the *Ta ta* males mentioned above. One daughter was produced in vial "*a*" (the first vial) by a heterozygous *Ta ta* female which ran through "*d*" (the fourth vial), producing also 58 males. This fertile daughter produced one type daughter which bred as a heterozygote, giving 10 tapering and 11 type males. From a second mating of this male with a tapering female there were obtained in "*a*" 18 tapering males and 2 sterile type females, in "*b*" 24 tapering males and 2 fertile type females and in "*c*" 13 tapering males. One of the fertile females produced one tapering male, the other produced 4 tapering males and 3 tapering females.

Since stung caterpillars were used with the tapering female of the second mating and for testing the daughters, contamination may explain the results. However, no cases of contamination were noted in other tests and 13 other daughters of *Ta ta* males were tested and found sterile.

The daughters of biparental males, in almost all cases, show only the dominant traits of their male parent regardless of how these traits entered his composition. In many cases one dominant was contributed by the male parent and another by the female parent of the biparental male and the daughter showed both of the traits.

It was interesting to note the appearance of characters determined by factors showing incomplete dominance. Daughters were obtained from o^d-o^i males mated to O-o^i and o^io^i females. The eye color of these daughters was about the same as that of biparental males or heterozygous females (o^d-o^i). Two *Yy* males produced daughters. One male was very dark; it was mated to a type (antennæ) female and produced 4 daughters with rather dark antennæ. The other was lighter; it was mated to a *Yy* female and produced a daughter having the 3 basal antennal segments clear yellow.

The small eye locus gave interesting results. Eighteen daughters were obtained from 3 matings. In 2 cases Kk^s males were mated to type females and produced 14 daughters, of which 4 had eyes much smaller than normal. In the other case, a Kk^s male was mated to a small eye female and the 4 daughters resulting had small eyes—much smaller than normal though not as small as small eye often is. This locus needs to be tested further.

For other factors (Table VIII), where dominance is known to exist, the daughters always showed the dominant traits of their male parent though he must also have carried recessives. These males, then, do not behave like diploid individuals; they do not segregate recessives. In order to explain this discrepancy it has been suggested that the sperm may be diploid.

If the sperm of biparental males are diploid, their daughters may be expected to be triploid. They must possess some irregularity in chromosomal constitution since they are often morphologically abnormal and rarely produce offspring.

The triploid nature of these daughters has been tested by means of sets of three pairs of factors, each dominant being brought in by a different individual. Two sets of such factors have been used.

In one instance the complementary eye color factors orange and cantaloup were used along with the factor for reduced wings. Seventeen biparental males resulted from the union of egg (*oCr*) with sperm (*Ocr*). These males had type (black) eyes and reduced wings. They (*OoCcrr*) were mated to orange cantaloup females (*ooccRR*). The 16 daughters from this cross were type showing the three dominants (*OCR*). This indicates probable triploidism.

More conclusive proof of the triploidy of the daughters was obtained by means of two linked (*Cc* and *Ll*) and one independently segregating (*Rr*) pairs of factors. Cantaloup (eye color) and long (antennæ, wings and legs) are linked and have a cross-over value of about ten per cent. Eighteen biparental males were obtained from crosses of cantaloup reduced (*cL/cL r/r*) females by long reduced (*Cl r*) males. The biparental males were type except for reduced wings and must, therefore, have been duplex for the *cl* chromosome. These reduced males *cL/Cl r/r*) were mated with cantaloup long (*cl/cl R/R*) females. One daughter was obtained. She has been thoroughly examined and compared with long and reduced wasps. One of her primary wings was not completely expanded, but camera lucida drawings were made of the other and of her antennæ and legs. These were compared with drawings of long. She was found to be type with respect to all three structures, showing that she possessed the dominant factor *L*. She was obviously non-reduced, *R*, and non-cantaloup (black-eyed), *C*, and hence must have resulted from union of sperm (*cL/Cl r/r*) with egg (*cl R*) and would, therefore, be (*cl/cL/Cl R/r/r*) triploid.

Since daughters of biparental males are often morphologically abnormal, rarely produce offspring and will inherit from three individuals in the manner shown above, it seems probable that they are triploid.

IV. Discussion

Data thus far obtained suggest that biparental males are duplex for all chromosomes studied. None of the seventeen mutations tested gave evidence of being simplex in biparental males and therefore located in a sex chromosome. Genetically we have as yet no sex chromosome and the question as to why biparental males are males is still unanswered.

Sex in *Drosophila* has been most conclusively shown to be dependent upon genic balance. Even the haploid has recently been shown (Bridges, C. B., 1930) to be female.

However, the situation in *Drosophila* must differ somehow from that in certain other forms where males are produced through haploid parthenogenesis and females are diploid. This apparently is true of most Hymenoptera (Whiting, P. W., 1918) and has been definitely shown to be true of many coccids. In *Icerya purchasi* it was found (Schrader, Franz and Hughes-Schrader, Sally, 1926) that, " the individual chromosomes of the haploid set as found in the cells of the males correspond in size to the individual chromosomes of the diploid,—one member of each of the two morphologically distinct pairs of the diploid set apparently being present in the haploid group." The same is true of other coccids (Hughes-Schrader, Sally, 1930).

Sex in such forms can hardly be based on the same sort of genic balance as in *Drosophila*. Bridges (1925) suggested that, " at present the difference between haploid and diploid sexes must be referred to the same type of determination as that responsible for the larger size, rougher texture of eyes and other slight changes that distinguish the 3 N from the 2 N individual," in *Drosophila*.

Castle (1930) postulates that there is no minus sex-tendency in species which have haploid males. " The egg is homozygous for plus sex tendency (XX) ; the haploid male transmits in its one class of sperm this same sex-tendency (X)." He is unable to state why these haploid individuals, genetically female, are phenotypically male.

One interesting point is the fact that there has been no indication of intersexuality in *Habrobracon juglandis* (Ashmead) though cases of intersexuality were noted in *H. brevicornis* (Wesmael) (Whiting, P. W., and Whiting, Anna R., 1927, p. 112). The more biparental males are studied, the more closely is their genetic composition found to parallel that of the diploid female. In spite of this genetic similarity to females the biparental males are as definitely male in morphology and reactions as are haploid males nor does their added chromatin make them noticeably larger than haploid males.

V. Summary and Conclusions

1. Biparental males inherited dominant allelomorphs, of eleven pairs of factors showing ordinary dominance and recessiveness, from both parents when reciprocal crosses could be made or from either parent carrying the dominant when they could not.

2. Biparental males inherited complementary dominant factors, one from each parent, in such a way as to reconstitute the type character.

3. Homologous chromosomes were shown to be present in biparental males by means of linked factors.

4. Four factor pairs which produce distinct heterozygous types were inherited by biparental males in such a way as to demonstrate a duplex condition in each case.

5. Daughters of biparental males were shown to inherit, in almost all cases, only the dominant traits of the male parent regardless of how these traits entered his composition.

6. The triploid nature of these daughters and the diploid nature of the sperm of biparental males were tested by means of three pairs of factors, each dominant being brought in by a different individual.

7. Biparental males appear to be diploid and their daughters appear to be triploid.

LITERATURE CITED

Bridges, C. B., 1925. Sex in Relation to Chromosomes and Genes. *Am. Nat.*, **59**: 127.

Bridges, C. B., 1930. Haploid Drosophila and the Theory of Genic Balance. *Science*, **72**: 405.

Castle, W. E., 1930. The Quantitative Theory of Sex and the Genetic Character of Haploid Males. *Proc. Nat. Acad. Sci.*, **16**: 783.

Dzierzon, J. Eichstaed, 1845. *Bienen*, Ztg. 1.

Hughes-Schrader, Sally, 1930. Contributions to the Life History of the Iceryine Coccids, with special reference to Parthenogenesis and Hermaphroditism. *Ann. Entom. Soc. America*, **23**: 359.

Schrader, Franz and Hughes-Schrader, Sally, 1926. Haploidy in Icerya purchasi. *Zeitschr. f. wiss. Zoöl.*, **128**: 182.

Torvik, M. M., 1929. Are Habrobracon Males Diploid for the X-ray Mutation "Short"? *Proc. Penn. Acad. Sci.*, **3**: 2.

Whiting, A. R., 1925. The Inheritance of Sterility and of other Defects Induced by Abnormal Fertilization in the Parasitic Wasp, Habrobracon juglandis (Ashmead). *Genetics*, **10**: 33.

Whiting, A. R., 1927. Genetic Evidence for Diploid Males in Habrobracon. *Biol. Bull.*, **53**: 438.

Whiting, A. R., 1928. Genetic Evidence for Diploid Males in Habrobracon. *Am. Nat.*, **62**: 55.

Whiting, P. W., 1918. Sex Determination and Biology of a Parasitic Wasp, Hadrobracon brevicornis (Wesmael). *Biol: Bull.*, **34**: 250.

Whiting, P. W., 1921. Studies on the Parasitic Wasp, Hadrobracon brevicornis (Wesmael). I. Genetics of an orange-eyed mutation and the production of mosaic males from fertilized eggs. *Biol. Bull.*, **41**: 42.

Whiting, P. W., and Whiting, A. R., 1927. Gynandromorphs and other Irregular Types in Habrobracon. *Biol.*Bull.*, **52**: 89.

THE CHROMOSOMES OF THE DOMESTIC TURKEY

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In a previous article (Biological Bulletin, Vol. LII, No. 5, May, 1927), I have described the chromosomes of the Indian runner duck, giving the probable number and forms, and have proposed a scheme for sex-linkage and sex-determination. The present study was undertaken in 1927 to find out whether or not the conditions found in the duck exist in other avian forms.

This study is based on the examination of approximately 800 mitotic figures taken from 35 individuals. The same four general methods of technique were employed as in the former work, except that in the technique for sectioned material of the testis, Bergamot oil was used instead of cedar oil. Since the tissues of the turkey were more difficult to prepare than those of the duck, the utmost precision was necessary in order to obtain desired results.

As in the duck, the cells of the male contain an even number of chromosomes, and the cells of the female an odd number and one more than is found in the cells of the male. The number of chromosomes in the turkey appears to be the same as in the duck.

In the embryonic tissues of the male one cell was found which appeared to have 66 chromosomes (Fig. 2), but a large majority of the cells examined contained 76 chromosomes. In the embryonic tissues of the female two cells were found which contained 55 chromosomes each. One of these is shown in Fig. 7. The remainder of the female cells examined appeared to contain 77 chromosomes each. It would appear that the typical somatic numbers are 76 for the male and 77 for the female.

As in the duck the chromosomes are of three forms: J-shaped, rod-shaped, and globe-shaped, and as in the duck the chromosomes fall into three general groups. In the male of the turkey these groups consist of 6 pairs of large chromosomes, 3 pairs of short rod-shaped chromosomes, and 29 pairs of globe-shaped chromosomes. In the female there are 6 pairs of large chromosomes, resembling in size and form those of the male, plus one odd chromosome which is the largest in the group; 3 pairs of short rod-shaped chromosomes; and 29 pairs of globe-shaped chromosomes. It is apparent that the difference in the chromosomal grouping in the duck and in the turkey is in the second and in the third groups. In the duck the second group contains nine pairs of short rod-shaped chromosomes and the third group contains 23 pairs of globe-shaped chromosomes.

In the aberrent cells of the male which contain less than 77 chromosomes (Fig. 2) all of the twelve large chromosomes of the first group are present, also the six short rod-shaped chromosomes of the second group. The missing chromosomes are the ten smallest of the third group. The same thing seems to be true in the aberrent cells of the female of less than 77 chromosomes. In these are present the 13 large chromosomes of the first group, the six rod-shaped chromosomes of the second group; but 22 of the smallest chromosomes of the third group are not present.

The same pliancy is noted in the chromosomes of the turkey as was evinced in the chromosomes of the duck. Because of this the chromo-

EXPLANATION OF PLATES

All figures from the turkey are reproduced at the same scale. The drawings were outlined with an Abbe camera lucida at a magnification of 3,500 diameters, obtained with a Spencer 1/12 homogeneous immersion objective and Spencer 15X compensating ocular with draw tube set at 150 mm., and drawing made at the level of the base of the microscope. The drawings were then enlarged by means of a copying camera lucida to 7,350 diameters. Having been reduced one-third in the reproduction they now appear at a magnification of 2,450 diameters.

W, the large sex-chromosome carrying female-tendency genes only.

w, the smaller sex-chromosome which also carries female-tendency genes only.

Z, sex-chromosome carrying a preponderance of male-tendency genes and also sex-linked genes.

38*Z*, same as *Z*.

38*w*, same as *w*.

37 to 1, autosomes.

Explanation of Plate I

Figs. 1–4. Cells from the amnion of males of the domestic turkey. The sex-chromosome is numbered 38*Z*. Autosomes from 37 to 33 are paired according to their size.

Fig. 1. Early prophase. The large chromosomes have not yet taken the characteristic peripheral position. Seventy-six chromosomes present.

Fig. 2. A soma cell aberrent in chromosomal number. This cell has the 12 large chromosomes of the first group, the 6 rod-shaped chromosomes of the second group, and 48 chromosomes of the third group. Ten of the smallest chromosomes of the third group are missing.

Fig. 3. Early metaphase showing gonomeric grouping of the largest chromosomes. Seventy-six chromosomes present.

Fig. 4. Early metaphase showing gonomeric grouping of the largest chromosomes, also some filamentous linkage between the members of the third group. Autosomes from 37 to 33 numbered. Seventy-six chromosomes present.

Figs. 5 and 6. First spermatocytes from smear preparations. These cells are in the prophase stage. The *Z* chromosome bivalent in each cell is numbered 38*Z*, the autosomal bivalents from 37 to 1 according to their size. Thirty-eight chromosomes present.

PLATE I

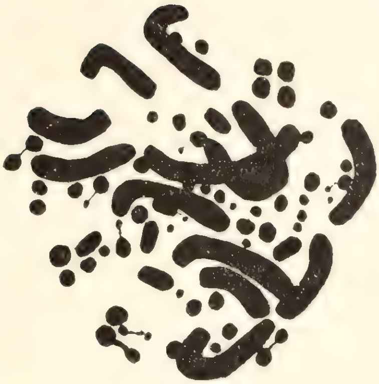


Fig. 1

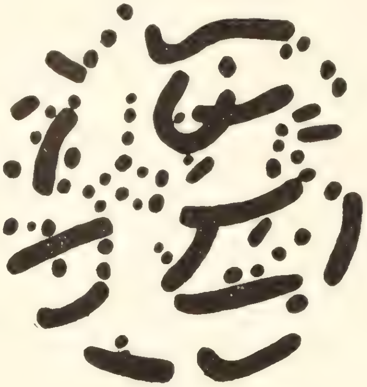
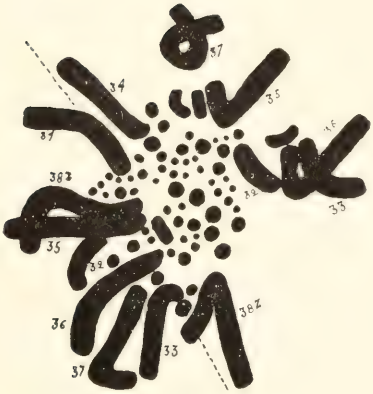


Fig. 2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34
35
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38z
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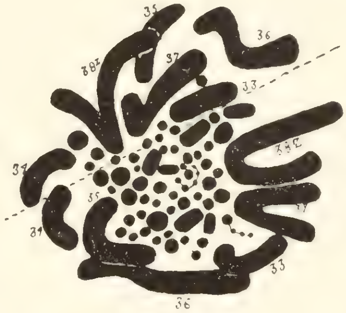
32
36
33
38z
37

Fig. 3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38z
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36

Fig. 4

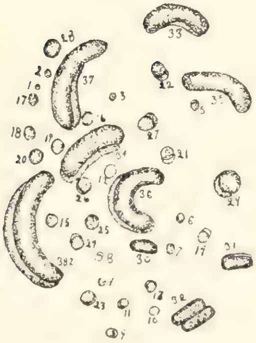


Fig. 5

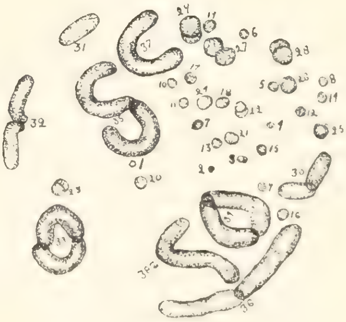


Fig. 6

somes are often found in more or less modified forms at the beginning of mitosis.

The seventy-six chromosomes in the complex of the soma cells of the male (Figs. 1, 3, 4) appear to form a graduated series from the largest of the first group, 38*Z*, to the smallest of the third group. Of the twelve large chromosomes which form the first group numbers 38*Z*, 37 and 36 appear in most cases as J-shaped and 35, 34 and 33 as rod-shaped. The six chromosomes which form the second group are all of the rod type. They are in most cases sufficiently smaller than the smallest of the first group so as not to be easily confused with them, but the slight difference in the length of the individual members of the three pairs makes it sometimes difficult to distinguish one from the other. The third group, the globe-shaped chromosomes, range in size from those containing approximately as much chromatin as the smallest of the second group to very small ones. Many of these small chromosomes are so nearly of the same size that they can be compared with no degree of certainty. Figures 5 and 6 are first spermatocytes from smear preparations of testis material. The tetrad form of many of the chromosomes is plainly apparent. It would appear that gonial mates have the same spindle attachment and that they are of the same size. In these cells the haploid number is present and the grouping of the chromosomes and the size relations are the same as in the diploid number. The germ cells are so small that in sectioned material it is practically impossible to be sure of the small chromosomes; but in most cases the large chromosomes are easily made out as to number and form. However, the testis material lends itself well to smear preparations and when the cells are well pressed out, the chromosomes are sufficiently large and clear to distinguish their number and form.

EXPLANATION OF PLATE II

FIGS. 7 to 12. Cells from the amnion of females of the domestic turkey. *W* and 38*w* are the chromosomes that carry female-tendency factors only. Autosomes paired from 37 to 33.

FIG. 7. A prophase aberrent in chromosomal number. There are present the thirteen large chromosomes of the first group, the six rod-shaped chromosomes of the second group, and thirty-six of the chromosomes of the third group. Twenty-two of the smallest chromosomes of the third group are missing. Fifty-five chromosomes present.

FIG. 8. Prophase. Seventy-seven chromosomes present. Some filamentous linkage shown. Seventy-seven chromosomes present.

FIGS. 9 to 12. Cells in metaphase. The stippled line in each case shows a possible gonomeric grouping. This grouping in each case has been considered with especial reference to the twelve largest chromosomes. Filamentous linkage of chromosomes shown in each cell. Autosomes paired from 37 to 33. Seventy-seven chromosomes present.

PLATE II

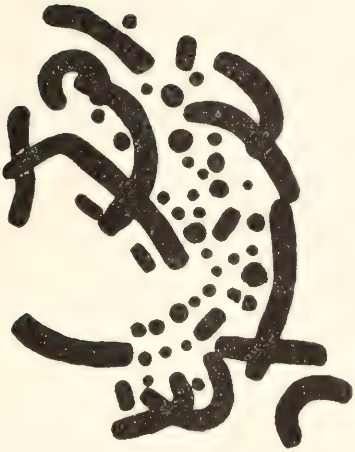


Fig. 7

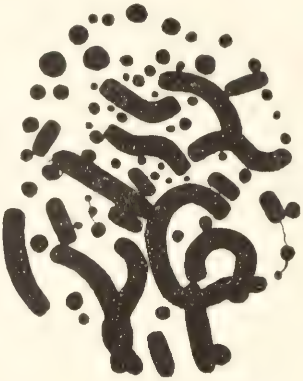
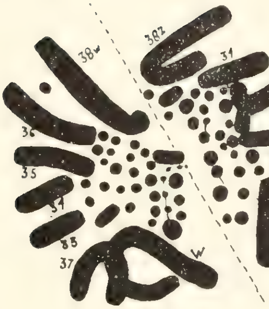


Fig. 8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38z
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33
36
37
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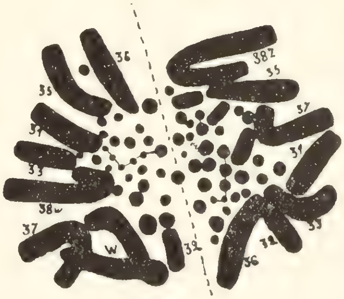
35
34
33
37
W

Fig. 9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38z
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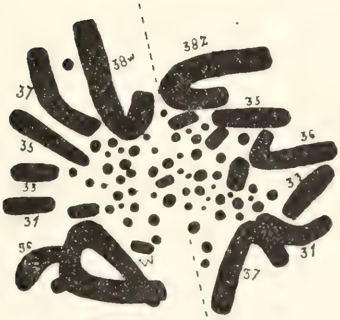
38w
37
W
32
32
36
33

Fig. 10


38z
38w
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36
33
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34
36
W
34
37

Fig. 11

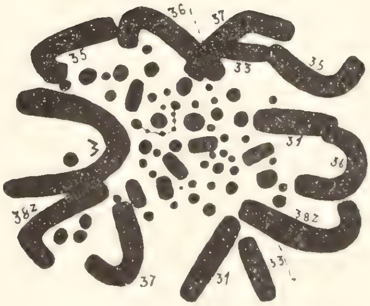
36
37
35
33
35
3
34
36
38z
38z
37
34
33

Fig. 12

As in the duck there is one more chromosome in the complex of the female than in the male. There are probably seventy-seven (Figs. 8–12). The odd chromosome is the largest in the complex. In these figures it is designated as *W*. This large chromosome, which is evidently a sex-chromosome, is quite pliant and apparently adjusts itself to other chromosomal regions and to the nuclear wall. For some time this chromosome was regarded as rod-shaped, but in anaphase it is J-shaped. However, it differs from the other J-shaped chromosomes in the complex in that the end that forms the loop of the *J* is tapering whereas the other J-shaped chromosomes are approximately uniform in diameter throughout their entire length. The next two largest chromosomes (38*w*–38*Z*) are apparently J-shaped. As in the duck it is difficult to say whether or not they are gonial mates. If the theory advanced in the former study concerning sex determination is correct then they are not gonial mates, but one of them must be regarded as a homologue of the 38's in the cells of the male and the other as a *w* chromosome. The remaining chromosomes in the complex of the female appear approximately the same as those of the same numbers in the cells of the male.

As in the duck gonomeric grouping is evident. In the metaphase of males (Figs. 3, 4) and in the metaphase of females (Figs. 9–12). As in the duck " . . . in the cells of the male the largest chromosomes are grouped six on one side of the forming equatorial plate and six on the other. . . . In the cells of the female there are six on one side of the plate and seven on the other. . . . In every case there is in the group of seven, one chromosome which is larger than the others which has the characteristic form of the largest odd chromosome in the cells of the female, large at one end and taper at the other." (Werner, Biological Bulletin, Vol. LII, No. 5, May, 1927).

I have not been able to determine whether the two halves of a nucleus are of exactly the same size. It would appear that in some cases there is some discrepancy in this respect. Neither have I been able to determine whether or not homologous chromosomes are of exactly the same size in any one stage of mitosis. In pairing the chromosomes I have selected as homologues those that are more nearly of the same shape and length. It is possible that there is a difference in the amount of chromatin material in some or all of the homologues. The chromosomes of the male may contain more than those of the female or vice versa. This is a difficult question but one that should be investigated.

As in the duck filamentous linkage occurs in the somatic cells. In most cases observed the linkage is between members of the third group, the globe-shaped chromosomes. (Figs. 1, 4, 8, 9, 10, 11, 12.) In other cases it was between members of the second group and some one member of the third group. (Figs. 1, 11). The numbers of chromosomes thus attached in linear arrangement range from two to seven. The filaments are in most cases one in number, although there are sometimes two. (Fig. 1). They are in all cases oxyphylic in character and are somewhat roughened or crinkly.

Discussion

The similarities between the chromosomal complexes of the duck and of the turkey are at once apparent. In each form there is in the male an even number of chromosomes, while in the female there is an odd number of chromosomes and one more than is present in the cells of the male. The same condition is found in the chicken (now being investigated). As in the duck it seems probable that the largest pair of chromosomes in the male complex are the *Z* or the sex-chromosomes. Since the large *W* chromosome has been found in the female of the three forms of the aves, it seems impossible to regard it as a planosome, or supernumerary. It must, then, be regarded as an odd chromosome and if such, it is reasonable to suppose that it is a sex-chromosome. Since it is found only in the cells of the female, it is evident that it is concerned only with femaleness. It is equally evident that it does not carry sex-linked characters but that this must be the function of some other chromosome in the female complex. The scheme proposed in the former article for sex-linkage and sex-determination in the duck is entirely applicable in the case of the turkey and it seems unnecessary to repeat it in detail in this article. The generalities are that the female tendencies are carried by the *Ww* chromosomes, the male by the *Z* chromosomes. In both sexes the sex-linked tendencies are carried by the *Z* chromosomes. The autosomes are in a balanced condition between maleness and femaleness. It follows that a zygote receiving a genic complex equally balanced between maleness and femaleness, plus that which contains genes for maleness only (the *Z* chromosome of the male) would of necessity become a male. A zygote receiving a genic complex equally balanced between maleness and femaleness plus the *Z* chromosome, which contains genes for maleness only, and in addition the *Ww* chromosomes which carry genes for femaleness only, would become a female. It would, of course, follow that the F_1 and F_2 generations would inherit as is usual in such sex-linkage and as has been outlined in the previous article.

Summary

1. The chromosomes in the somatic cells of the turkey agree in number with the chromosomes in the somatic cells of the duck. These "appear to be 76 chromosomes for the male and 77 chromosomes for the female. There is present in the cells of the female a long unpaired chromosome which is not found in the cells of the male. There is reason to suppose that there are probably among the remaining six largest chromosomes two more unpaired chromosomes, one of which, the largest, is probably homologous to the largest pair (sex-linkage) of chromosomes in the male complex, while the other, it is thought may be some one of the five remaining long chromosomes." (Werner.)

2. As in the duck the 76 chromosomes appear to fall into three general groups. In the duck these groups consist of six pairs of large chromosomes, including three J-shaped and three rod-shaped; nine pairs of short rod-shaped chromosomes; and twenty-three pairs of globe-shaped chromosomes. In the turkey the first group consists of six pairs of large chromosomes, including four pairs of J-shape and two pairs of rod-shape. The second group consists of three pairs of short rod-shaped chromosomes; the third group consists of 29 pairs of globe-shaped chromosomes, which as in the duck, form a closely graduated series.

3. As in the duck there appear to be 38 bivalents in the primary spermatocytes of the male. These agree with the somatic cells in size gradations.

4. Gonomeric grouping occurs in the amnion cells as it does in the duck.

5. Filamentous linkage occurs in certain stages of the prophase and metaphase. This also agrees with the condition found in the duck.

6. The sex-mechanism appears to be of the WwZ–ZZ type similar to that found in the duck and in the moth *Phragmatobia*.

I desire to record my indebtedness to Dr. W. R. B. Robertson for the material for this work and for his criticism of the major part of the work; to the Bausch and Lomb Optical Company for the use of microscopic equipment during the year 1929; to Dr. Mary Rose Prosser, President of Cottey College, and to Mrs. Elizabeth Ott for their influence in securing from the Spencer Lens Company the proper equipment for the completion of the work.

THE SIGNIFICANCE OF HYDROGEN ION CONCENTRATION IN THE BIOLOGY OF EUGLENA GRACILIS KLEBS

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INTRODUCTION

Euglena gracilis Klebs is a common and important constituent of certain aquatic communities, but is easily cultured under laboratory conditions. Hence, it is especially suitable for investigations in the ecology of a single species, and the present study is a contribution to that field. As Allee (1930) pointed out in his presidential address before the American Society of Ecologists, we do not begin to know as much about the morphology and physiology of individual species as is desirable, and the mere cataloguing of organisms from different environments has yielded little of real value.

The present study is designed to show the effects of different H^+-ion concentrations in the external medium on *Euglena gracilis,* with other factors controlled in such a way that indirect effects, or effects from unknown variables, are reduced to a minimum. In nature, probably most of the effects of H^+-ions are indirect, but we can discover their true nature only by eliminating them under controlled conditions. For this reason, in the present studies, cultures free from all other organisms have been used.

Certain aspects of the physiology of *Euglena gracilis* are fairly well known, due to the researches of Klebs (1883), Zumstein (1899), Ternetz (1912) and others. In particular, its tolerance of high concentrations of citric and other acids was pointed out by Zumstein and Ternetz, both of whom made use of citric acid in the more or less complete elimination of bacteria from cultures of the *Euglena.* Kostir (1921) demonstrated that this high degree of tolerance for citric acid is, however, a species characteristic not generally true for the genus.

Zumstein emphasized the necessity of using bacteria-free cultures, asserting that the presence of bacteria materially depressed the division-rate. He believed that the increased division-rate in acid cultures was not a direct effect of the acid, but an indirect effect, through the elimination of the bacteria. His evidence was incomplete, but my own experiments demonstrate that his view was correct.

OGICAL LA RATORY MASS.

It is possible that other organisms may be similarly effective. Skadowsky (1926), studying cultures of mixed Protozoa in relation to H^+-ion concentration, arrived at an optimum pH for *Euglena gracilis* Klebs of about 3.8, a value very different from that which I have found in pure-line sterile cultures but not far from my finding in a series of cultures in which bacteria were present. Other Protozoa in Skadowsky's cultures showed different pH optima, his results indicating, for those species listed, almost no competition at optimum values. *Euglena gracilis* actually grows well in a wide pH range, as will be shown later, with a not very pronounced optimum. Therefore, may not this apparent (and very striking) optimum found by Skadowsky be due to the competition of one or more other forms at the true optimum for this species, depressing the division-rate of the *Euglena* below that at which it has no competition? Interspecies competition is very real, certainly applying to Protozoa as well as higher forms; and, in a case like that in question, it may mask the real responses of the individual species. The results obtained by Skadowsky may well apply, therefore, to *Euglena gracilis* in the community which he studied, but not to this species when isolated from others. The responses under the later conditions are fundamental to the particular species considered, but subject to modification by the presence of other forms. In studying the responses of an organism to an environmental factor under natural conditions, one must remember that the results of such findings apply only to the special complex community in which the study is carried out.

As far as the writer is aware, no observations of the present nature have previously been carried out on any of the Euglenoidina. A few related studies on the alga, *Chlorella*, have been made by Warburg (1919) and Wann and Hopkins (1927). A very good summary of studies on pH in relation to Protozoa, ciliates in particular, is that of Darby (1929), whose observations are extended in a later paper (1930). The earlier paper contains a good bibliography. A recent paper of general interest, which emphasizes the significance of CO_2 in influencing the pH of natural waters, is that of Powers (1930). A most satisfactory summary of the relations between pH and fresh-water and marine organisms is that by Bresslau (1926). A long bibliography is appended. Skadowsky's paper is of similar general interest.

Thanks for special favors in connection with the present study are due to Dr. W. B. Baker, of Emory University, for verification of my identification of the first specimens used; to Dr. C. H. Philpott, of Harris Teachers' College, for a demonstration of the technique of sterilizing *Paramecium;* and to the Digestive Ferments Company, of Detroit, Michigan, for information connected with the analysis of their product, "Bacto-peptone." To Professor E. Newton Harvey, I am especially obligated for continuous advice and assistance in technical aspects of the problem, to say nothing of the inspiration gained from association with him in his laboratories.

EXPERIMENTAL METHODS

The apparatus and methods of procedure used were, of course, necessitated by the desire to maintain unvarying from culture to culture all important factors, except pH, in the growth of *Euglena gracilis*. These factors are not only those affecting growth or reproduction, but also the limiting factors for photosynthesis. (Blackman, 1905; Stiles, 1925; Spoehr, 1926.)

The following physico-chemical conditions were subject to control: Temperature; frequency and intensity of light; a sufficient quantity and quality of food for both saprophytic and holophytic nutrition; in certain cases, carbon dioxide and oxygen tensions; hydrogen ion concentration.

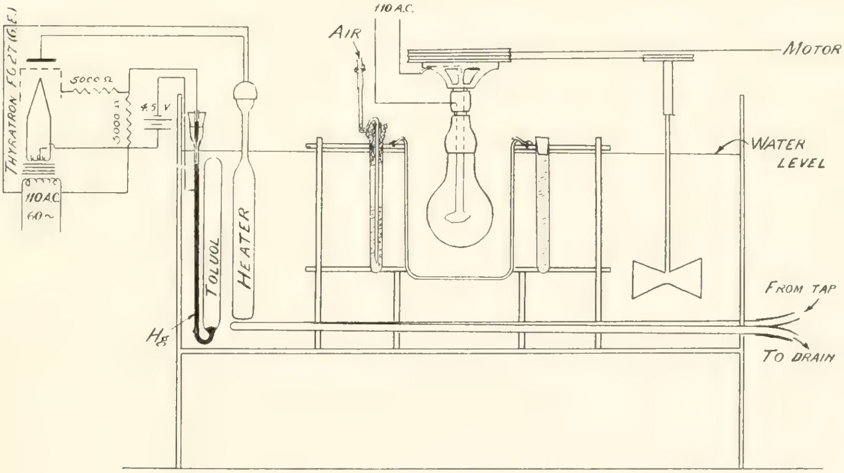

FIG. 1. Diagram of apparatus used in experiments.

Biological factors considered were: Absence of (1) a complicated life-cycle, (2) racial differences, and (3) other organisms; uniform density of organisms in all cultures at the beginning of an experiment; uniformity in organisms used for inoculation,—secured by using stock cultures of the same age and pH (in all but one experiment with etiolated *Euglena*).

For temperature control a water thermostat was adopted. This is illustrated in Fig. 1. The container is a metal tank, all inside parts painted black. The temperature balance is maintained between a loop of copper pipe through which tap-water flows, and a knife heater. (A significant amount of heat is also derived from the illuminating source.) The regulation is by means of a Thyratron tube in circuit with a toluol-mercury thermo-regulator, as described by Loomis, Harvey and MacRae (1930). Temperature fluctuations, as determined with a Beckman thermometer, are less than 0.01° C.

As a source of light a 60-watt Mazda Daylight Lamp (frosted) was used. This was rotated about its vertical axis (at about 150 revolutions per minute) to provide uniform total distribution of radiation in all directions. However, since experiments with a 40-watt lamp resulted in rates of division as high as those with the 60-watt, the light intensity used was not limiting photosynthesis. As controls for the detection of photosynthesis and other light effects, identical cultures in absolute darkness were maintained in all experiments. The culture-tubes were covered with several coats of black varnish, and, further, separated from the source of light by an opaque metal screen.

In the tank the water was kept in constant circulation to maintain uniformity of temperature. The lamp was not suspended directly in the water, but inside a Pyrex cylinder closed below and open above (a beaker was used). Much of the heat from the lamp was conducted away in the air. Between this Pyrex wall and the cultures (in Pyrex test-tubes 18 × 150 mm.,) was a water thickness of 5.2 centimeters. The total distance from the axis of rotation of the lamp to the center of each test-tube was 12 centimeters. Distilled water was used in the tank, to avoid deposition of films of carbonate on the glassware. The test-tube rack is of aluminum, painted black.

Temperature is known to have a marked positive effect on rates of photosynthesis; but death from high temperature is accelerated in high concentrations of H^+-ions, as has been shown by Chalkley (1930) for *Paramecium*. In a preliminary experiment with *Euglena gracilis* I have observed the latter phenomenon at temperatures as low as 35° C. In the selection of a suitable temperature for the experiments one must, therefore, compromise. I have actually used temperatures of 29° and 29.5° C.

Bacto-peptone, "Difco" Standardized, was selected as the basic culture medium. Previous workers have used "peptone" chiefly, a fact which makes my observations the more comparable with theirs. Furthermore, this medium is not only sufficient for saprophytic nutrition, but also permits photosynthesis without the addition of other media, —at least between pH 8.5 and 3.5. In addition, Bacto-peptone is in itself a very well buffered system, and is therefore especially suitable

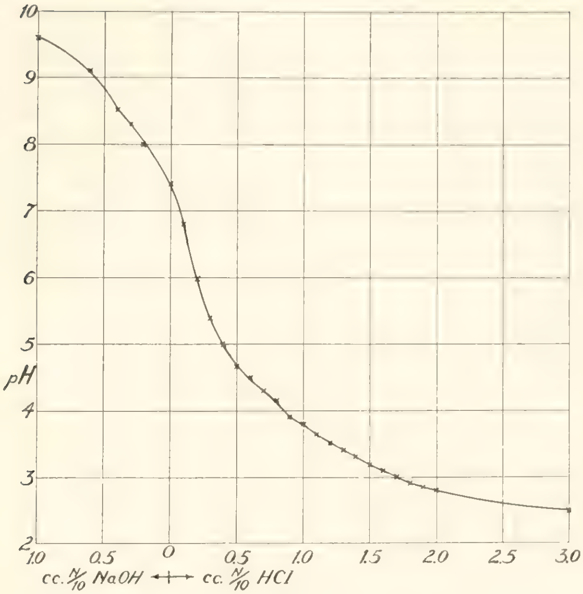

FIG. 2. Buffer curve for 1 per cent Bacto-Peptone in distilled water.

The original volume, to which were added the indicated quantities of NaOH and HCl, was 10 cc.

for studies of H^+-ion concentration (Fig. 2). Even within the pH range at which the buffer action is least, the pH changes produced by the growth in it of *Euglena* are, during the periods of experiments, not great (Table I).

The concentration of peptone used—dissolved in glass-distilled water—has been 1.0 per cent. With the density of organisms studied, no

TABLE I

Change in pH of cultures during an experiment lasting 48 hours.
These figures are from the experiment plotted in Fig. 5.

Initial pH	Final pH	
	In Light	In Darkness
8.10	8.13	8.07
7.65	7.72	7.70
6.71	6.87	6.87
5.90	6.11	6.15
4.60	4.64	4.84
3.56	3.55	3.55
2.96	2.81	2.89
2.52	2.39	2.48

greater growth is observed in this concentration than in 0.5 per cent, but its buffer value is somewhat greater.

The different pH values studied were produced by additions of hydrochloric acid, an acid probably completely dissociated, and which hardly, if at all, penetrates living cells. Its anion, furthermore, is such a common constituent of living matter as to be a relatively insignificant factor. The culture medium was always freshly prepared, and autoclaved but a single time for twenty minutes at fifteen pounds pressure.

This peptone is free from carbonates or bicarbonates in detectable quantity, and as a source of CO_2 for the organism it furnishes no more than that produced by the oxidation of the food-stuffs of which it consists. The medium is, therefore, free from a source of CO_2 that might be released with increased H^+-ion concentration. In any case, with peptone as the culture medium, the experimental results indicate that the major growth of *Euglena gracilis*—even in the light—is not due to photosynthesis, and is, therefore, not dependent on a supply of carbon dioxide.

The oxygen-tension, while apparently not a significant factor in the decomposition of CO_2,[1] is a very important factor in saprophytic nutrition. In cultures sealed from the air, the photosynthesis of the contained organisms provided the oxygen supply,—a supply which varied with the extent of photosynthesis. The greater the rate of photosynthesis, the greater the oxygen production, and, presumably, the greater the growth due to the oxidation of the organic foods in the medium. In aërated cultures, on the other hand, the organisms always had available a supply of oxygen (as well as carbon dioxide) in more or less complete equilibrium with the air. In cultures only initially aërated, and with access to the air at the surface, growth was somewhat limited by the oxygen-tension; but, in several experiments, a constant saturation with air was maintained.

The only factor intentionally varied from culture to culture has been the H^+-ion concentration. The range studied with most care has been between the limits of about pH 8.5 and 2.4, but the absolute limits of life have been approximately determined.

For measuring H^+-ion concentration, the quinhydrone electrode method was adopted. It is dependable to 0.02 pH, or less, below a pH of about 8.5, and is quite as convenient and rapid in use as the colorimetric method,—at least for pigmented solutions. By this method it is possible to determine with accuracy such pH changes as occur in an active culture during the period of an experiment, the direction of change giving a clue to the occurrence or absence of photosynthesis. The values were recorded to 0.01 pH.

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[1] Harvey (1928) has shown that decomposition of CO_2 can take place in absence of oxygen. The method which he used with marine algæ, *i.e.*, luminous bacteria, as the indicator, was applied to *Euglena gracilis* with results similar to those obtained with the algæ.

The stock cultures were grown in peptone medium unmodified by the addition of acid (pH between 7.2 and 7.9), with continuous artificial illumination, at room temperature. The chlorophyl content of the organisms did not vary materially from culture to culture. One study, however, was made with etiolated *Euglena* from a stock culture of a lower pH. The differences in division-rate between the green and etiolated ones were not great. Hence, slight differences in the chlorophyl content could not make appreciable differences in the results —except when using inorganic media alone.

Because of the so-called allelocatalytic effects of Robertson (1922), or a decreasing division-rate with increasing numbers (Jahn, 1929), it is necessary to compare cultures which begin with approximately the same numbers of organisms. By using for inoculation stock cultures that, to the eye, appear to be of about the same density, one can approach this condition. In any one experiment, each culture was inoculated with the same volume of stock culture. The variation in numbers per unit volume, as determined by actual counting in every case, was less than 10 per cent.

For counting, I have used a Rafter Counting Chamber, 1.0 cc. capacity, with a squared disc in the microscope ocular. Before counting, the organisms were killed and allowed to settle on the bottom. They were killed by heating to 65° C., a temperature at which they are coagulated but do not disintegrate under any of the observed conditions. The counts by this method were consistent to under 10 per cent (totals of ten counts each, from different parts of the chamber), even in cultures of lowest density of population. The error in the initial count probably determines the total error in counting. It is not greater than 10 per cent.

The chief source of error in the present experiments is in this determination of the numbers of individuals at the beginning and end of "runs." Slight differences in temperature, light, CO_2-tension, and other physico-chemical factors, would probably not produce variations greater than those between different counts of the same culture. The possibility of deviation from the true results is 10 per cent on the basis of counts alone, this probably determining the error in the experiments. We are not justified, therefore, in emphasizing any differences between cultures unless they are of a greater order of magnitude than about ten per cent—though consistent differences of less than that are probably real.

Genetic variables have been eliminated as far as possible by the use of organisms all descended from a single ancestor—a pure-line or clone. Variation due to complicated life-cycles has not been demonstrated in *Euglenæ.* (Reports of conjugation have been received with much scepticism.) Encystment occurs, but it was not observed in the course of any of my studies on pH. I found cysts in old stock cultures, of course, but none in the test-tube cultures after the two, three or four days of my experiments. Encystment is, therefore, not induced by any pH within the range 8.5–2.4. This is rather interesting, for encystment of some ciliates seems to be related to pH (Koffmann, 1924; Darby, 1929). I have found no evidence for a regular cycle involving encystment in *Euglena gracilis,* nor am I aware that any previous workers with this species have found such a cycle. Reproduction is, according to my observations, due solely to longitudinal fission. In the present study, the rate of reproduction is taken as the chief criterion of the effects of pH. This rate has been determined by considering the initial and final counts in a culture the first and last terms of a geometric progression with two as the common ratio.

The method used in establishing a *sterile,* pure-line culture was that used by Hargitt and Fray (1917) and Philpott (1928) for *Paramecium.* The parent individual for the present experiments was isolated October 2, 1930 and washed by transfer from one to another of five drops of sterile medium, being left in each for two or three minutes.[2] This individual and others sterilized at the same time were separately transferred to test-tubes, each containing about twenty cubic centimeters of sterile medium. Each culture began to appear greenish within about a week. Absence of other organisms was verified by making plate cultures on nutrient agar. The *Euglena* appeared on the agar, and reproduced rapidly, but no other forms were observed.

Experimental cultures were maintained sterile by ordinary methods of bacteriological procedure. Cultures sealed from the air were closed with sterilized, paraffined corks, tinfoil covers for corks having proved toxic in preliminary experiments. Air bubbled through sterile cultures was always sterilized by first passing it through sterile cotton, as illustrated in Fig. 1.

RESULTS

Preliminary studies demonstrated that the previously reported increase in numbers of *Euglena gracilis* with increased concentrations of citric acid was largely related to the H$^+$-ion concentration. Maximum growth in cultures made up with either hydrochloric, sulphuric, oxalic or citric acid occurred at pH 3 to 4, if no sterile precautions were observed. At higher pH values bacteria were numerous, especially near neutrality, but at the " optimum " few were present. Media adjusted to pH 3.6–3.7 by valeric or salicylic acids were fatal to *Euglena gracilis,* although the organisms thrived in more acid cultures prepared with the other four acids. The easily penetrating acids, as one should expect, proved fatal. The effect of the other acids was, however, obviously related to H^+-ion concentration, being independent of the kind of acid used. Subsequent experiments have shown that the increased growth at low pH is not a real effect of pH, but is an indirect result of the elimination of bacterial competition at these values.

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[2] Parpart's criticism (1928) of the method of Hargitt and Fray does not apply in the case of *Euglena,* since it is extremely doubtful if *Euglena* ever ingests solid food.

In studies on a sterile pure-line, carried out in 1925, the maximum division-rate when CO_2 was the only acid added occurred at about the same pH of the medium as when hydrochloric acid is used,—*viz.,* pH 6.5 to 6.9. Concentrations of CO_2 above that represented by 5 per cent saturation at room temperature were not accompanied by increased division-rates under the conditions of the experiments. From these early observations I saw the desirability of, first of all, determining the relations between pH, as such, and the rate of reproduction of *Euglena gracilis.*

Sealed Cultures

Typical results from a series of sealed cultures are plotted in Fig. 3. Not much weight can be placed on the irregularities in the figures from the controls in darkness, but we can say that no striking effects are observable. The exhaustion of the limited oxygen supply, and the impossibility of its replenishment, make continued growth impossible at any pH in the dark.

An interesting collateral experiment was undertaken in this connection to determine whether or not this *Euglena* can live under anærobic or near-anærobic conditions. Similar cultures, in tubes with capillary necks, were exhausted as completely as possible with a vacuum pump. Each culture tube was then sealed at the neck in a flame. One set was placed in darkness, the other, in the light. Fairly rapid reproduction occurred in the latter. In the culture in darkness, however, the organisms were all motionless within twenty-four hours, and within another day were all encysted. They died in the encysted condition, for, when air was readmitted by breaking the sealed tip they did not excyst, nor did they excyst when transferred to fresh medium.

These results may be explained in the following way: In both cultures traces of oxygen and carbon dioxide remained after the exhaustion. In the light, a trace of the latter would be sufficient to initiate photosynthesis. This process once begun, the *Euglena* would be self-sufficient for both oxygen and carbon dioxide. But in darkness, after the little oxygen present had been used, the organisms were in complete absence of oxygen—physiologically—and could not replace it. Incidentally, it is of interest to note that this is the only means I have yet found to produce encystment in this form.

From this collateral experiment we are able to say that the low division rates in darkness, as represented in Fig. 3, were probably due to the decreasing oxygen tension. It is doubtful if this effect is correlated with H⁺-ion concentration.

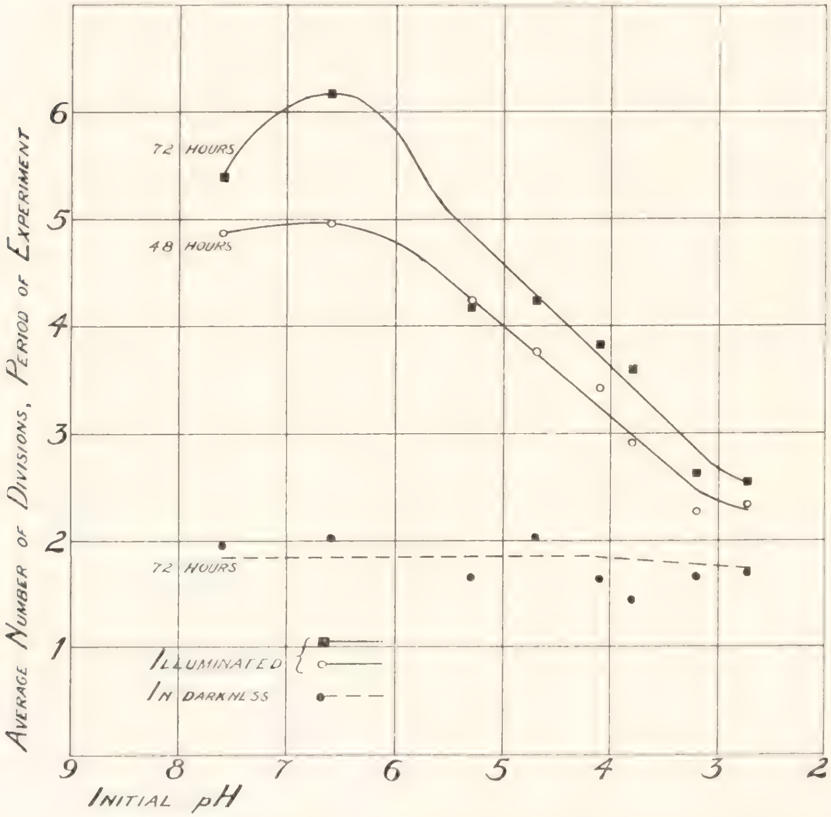

FIG. 3. The relation between initial pH and rates of division in sealed cultures.

In the light, on the other hand, a pronounced optimum at about pH 6.7 was apparent in all series. (This optimum has been, in another experiment, definitely established as below pH 6.9 and above pH 6.2.) The difference in rate between growth in light and in darkness must be due either directly to the products of photosynthesis or to the increased oxygen supply available from the decomposition of CO_2. Since this difference is much less marked in aërated cultures, as will be shown later, it is obvious that most of the increased growth in the sealed cultures is only indirectly due to photosynthesis. It is actually due to the oxidation of foods in the medium, but is made possible by the oxygen produced during photosynthesis. *Euglena,* combining as it does two forms of nutrition, uses more oxygen in its normal metabolism than does a completely holophytic organism—the latter always producing, in good light, more oxygen than is required in its own metabolism. If this latter statement were not true, of course, life on earth would be impossible.

Reproduction is fairly rapid between pH 7.6 and 4.0, but there is a pronounced decrease in rate on both sides of the peak,—the rate at the peak being as great as in initially aërated cultures. Photosynthesis is, obviously, going on wherever growth in the light exceeds that in the dark, and this difference in rate is proportional to the amount of photosynthesis. Therefore, it is apparent that, under conditions stated, a definite optimum for photosynthesis exists in this form, and that it is near pH 6.7. Furthermore, the oxygen production at this pH is great enough to maintain reproduction at near the maximum rate observed in media in equilibrium with the air.

While making the counts at the end of the experiments just described, I observed in the most acid cultures a considerable number of organisms attached to each other in pairs. The point of attachment was the posterior tip, the last part to divide in longitudinal fission. I repeated this observation with a stock culture, of pH 2.9, heavily inoculated with *Euglena,* and maintained under constant illumination. This was first examined after forty-eight hours. At this time many of the organisms present were attached in pairs, as previously described, but, in addition, there were groups of three and four individuals composing these multiple monsters—and always all individuals were joined at the same point, the posterior tip. Table II gives their relative numbers in samples of uniform volume counted at the end of forty-eight hours after inoculation. Figure 4 consists of camera lucida drawings of several of these, and one or two other abnormalities which appeared with them. In every case in which the individuals were of approximately equal size, the connection was purely at the surface—no cytoplasmic connection being observable even with most careful examination under oil immersion.

The " colonies " of three or more individuals assumed the shape of rosettes, in appearance reminding one of small colonies of colonial algæ.

TABLE II

Relative Numbers of Single Individuals and Multiple Monsters in Ten Equal Volumes, from a Culture at pH 2.9 Examined 48 Hours after Inoculation

Single Individuals	Multiple Monsters		
	Double	Triple	Quadruple
24	11	1	
25	13		
16	12	1	
21	9		1
23	9		
19	8	4	
28	17	1	1
23	18	3	2
23	7	1	
23	14		
Average 22.5	11.8	1.1	0.4

Their progress through the medium was very irregular, depending apparently on the resultant of the vectors represented in the aggregate. Groups of as many as six individuals were observed, always attached at the point last to divide in their typical form of reproduction.

The effect is entirely on the surface, and is dependent on the H^+-ion concentration,—since it always occurs to a greater or lesser extent below a pH of about 3.5, and is equally common in illuminated or darkened, sealed or aërated cultures. It is apparently permanent. Subsequent

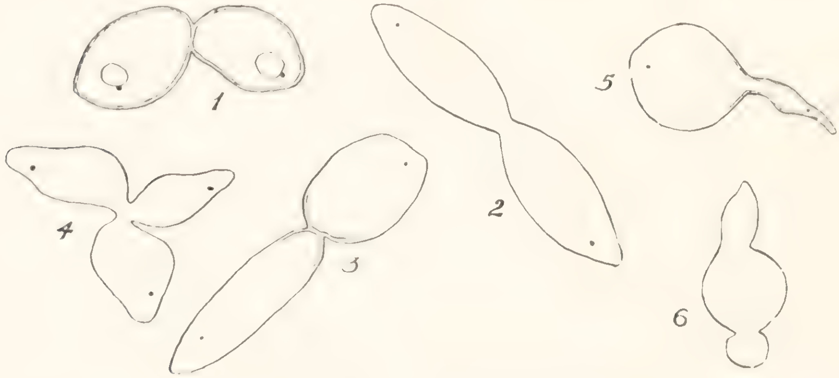

FIG. 4. Camera lucida drawings of monsters associated with media of low pH. 1–3, typical double monsters; 4, a triple monster; 5–6, other types of abnormalities observed. In 5, the cytoplasm was continuous between the "bud" and the parent organism.

examinations of the stock culture showed that most, if not all, of these multiple monsters never separated into individuals, but sank to the bottom and died in a few days. The cultures slowly developed, however, presumably habilitated by those individuals that had not been affected.

As the culture began to assume again the appearance of life (there was a period of a few days in which, to the eye, it seemed to have died out) an additional morphological effect of low pH began to be evident. The organisms now developing in the culture could be distinguished, but were not green. Examination under the microscope showed apparently complete etiolation, commonly extending in part to the stigma. The stigma was much reduced in size,—indistinguishable, in fact, in some individuals. Although several causes of etiolation have been found in *Euglena gracilis*, that due to low pH has not been previously reported.

Zumstein brought about etiolation by keeping cultures in an organic medium in complete darkness. I have repeated this, using sterile pure-line cultures. Zumstein also stated that the green form became colorless in " very rich organic medium." Ternetz described another hyaline form, differing from those observed by Zumstein in being permanently colorless. This form had completely lost the stigma, as well as chlorophyl. The type of etiolation associated with low pH is, however, not permanent. It may be similar to that caused by darkness or a " very rich organic medium," or may be identical with the latter, or with both. In any case, inoculation of culture medium at pH 7.2 with etiolated organisms from that at pH 2.9 yielded a normally green culture in a little more than a week.

At about this same time a stock culture was prepared to use in a study of the possible effect of initial adaptation in changing the shape of the pH—division-rate curve. This culture was adjusted to pH 4.6. As in the more acid cultures etiolation took place. When used for inoculation, the culture did not appear green to the eye, but when held to the light showed a rich growth of organisms. Under the microscope these individuals appeared colorless.

I have no explanation to offer as to the mechanism of this effect, but it is certainly associated with a high concentration of H^+-ions. Etiolation of *Euglena gracilis* ordinarily occurs in darkness at any pH, but, even in the light, and with continuous illumination, it occurs below pH 5 ±. This etiolation, either in darkness or at low pH in the light, is evident on microscopical examination after only forty-eight hours.

Aërated Cultures

The next experiments were designed to provide a sufficient supply of oxygen in both light and darkness. Figures 5 to 7, inclusive, represent typical results,—the two former under conditions of initial aëration only, and Fig. 7, under conditions of continuous aëration as compared with initial aëration only, in identical cultures inoculated at the same time.

Air from a storage tank was bubbled through the cultures at a vigorous rate, with shaking, for two minutes. The cultures were in flasks, each containing 80 cc. of medium and each adjusted to a particular pH. The aëration was carried out immediately after the introduction of organisms for inoculation. Following aëration, most of each culture was divided among three or four sterile test-tubes plugged with cotton, each containing 15 to 20 cc.,—two of these to be illuminated, and the other or others for a control in darkness. (During the experiments these cultures had access to the air through cotton plugs.) The pH was then determined in the medium remaining, and the final portion was used for determining the density of organisms. Each culture was shaken just before each transfer in order to maintain uniform distribu-

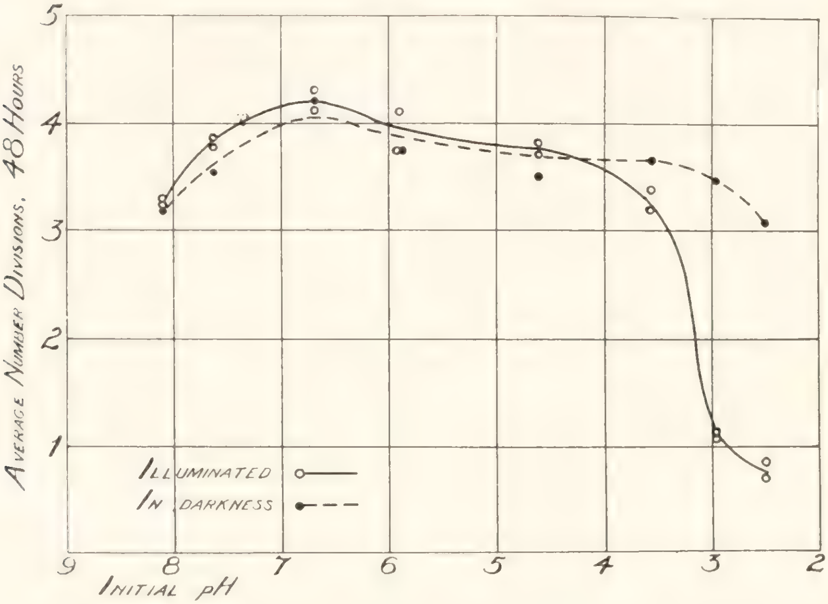

FIG. 5. The relation between initial pH and rates of division of normal green *Euglena*, in cultures initially aërated, and exposed to the air through cotton plugs.

tion of the contained organisms. Agreement in counts from culture to culture was very close, and, since the initial volume of inoculating culture was the same in each, this indicates that the samples counted fairly represented the cultures used in the experiments.

The results from the initially aërated cultures are very different, both under illumination and in darkness, from those obtained from sealed cultures. In the experiment plotted in Fig. 5, in which normally green organisms were used, there was, above pH 4, a surprisingly small difference in division-rate between those in light and those in darkness. This, of course, is conclusive evidence that the major growth of this *Euglena* in the medium used is dependent on saprophytism, and not photosynthesis. However, there is a slightly greater growth in the light between pH 8.1 and 4.6. This is less than 10 per cent greater; but in another experiment I found a difference of 15 per cent in the same direction, at pH 7.5 and 6.6, under conditions otherwise the same. It is probably, therefore, a real difference, and indicates that photosynthesis plays some part in growth.

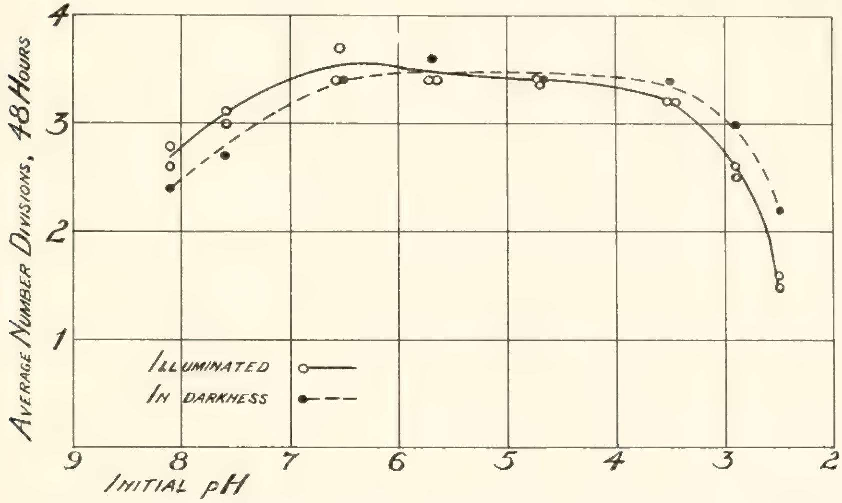

FIG. 6. The relation between initial pH and rate of division of etiolated *Euglena,* in cultures initially aërated, and exposed to the air through cotton plugs.

At about pH 4, on the other hand, the curve of growth in the light falls away rapidly, whereas that of cultures in the dark remains at a fairly high level. This indicates the presence of a photodynamic compound.

Comparison of Fig. 5 with Fig. 6 gives a clue to the nature of this compound. In Fig. 6, the results of experiments begun with etiolated individuals are recorded. At the alkaline end of the range there is, again, evidence of photosynthesis. This is not unreasonable, for examination of the *Euglena* under the microscope showed the presence of small quantities of chlorophyl, even after only forty-eight hours in the cultures. On the other hand, there is no evidence of photosynthesis below pH 6. There is some evidence of a photodynamic effect toward the acid end of the range, but it is not nearly so pronounced as in the series begun with green organisms. This suggests that the photosensitizing material is either chlorophyl or one of its derivatives. Since etiolation occurs at about the same pH values, we cannot be certain that the chlorophyl itself is the material. In any case, it seems strange that an organism could be made photosensitive by one of its normal constituents. As far as I know, this is the first report of this effect. A photodynamic effect of chlorophyl (production of hemolysis of mammalian erythrocytes) has been demonstrated by Hausmann (1909), but

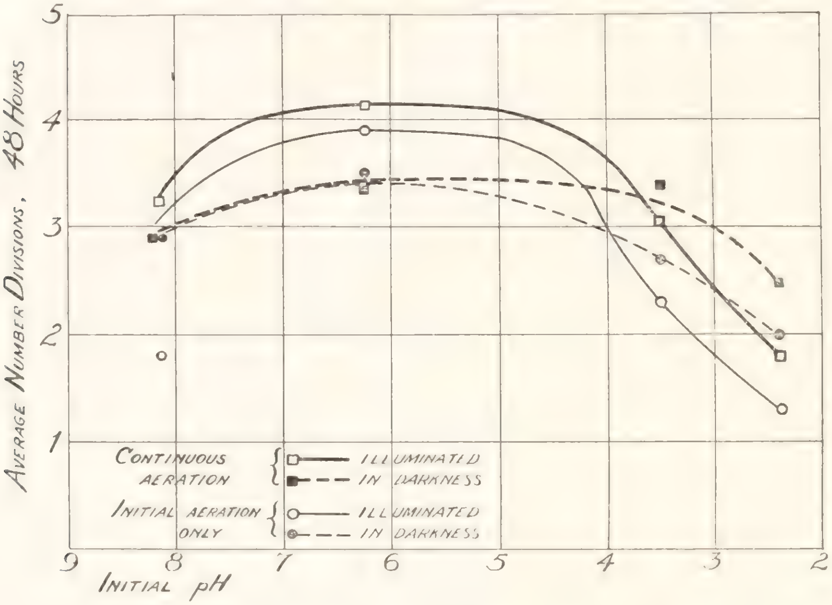

FIG. 7. The relation between initial pH and rates of division in cultures continuously aërated compared with that in cultures only initially aërated but subsequently exposed to the air through cotton plugs.

The flat top of the curves representative of continuously aërated cultures is based on another experiment, in which maximum rates were observed also between pH 7 and 8, and between pH 5 and 4. For convenience, only four pH values were selected in each "run." The very low rate of the initially aërated, illuminated culture at pH 8.1 + was due to bacterial contamination.

a comparison with the observation herein recorded seems rather far-fetched.

The stock culture used to inoculate the series plotted in Fig. 5 was at pH 7.9, that used in the experiment with colorless *Euglena,* at pH 4.7. In spite of this initial difference in pH, no very evident adaptive effect of the low pH in the stock culture is apparent. The curves are not strikingly different except at the extreme acid end of the range, and there the principle difference occurs only in the light.

As in sealed cultures, the undivided, multiple organisms appeared in the more acid conditions.[3] In one of these initially aërated cultures the group of six, mentioned previously, was observed. This group can have evolved as follows: The first individual divided partly, forming individuals a and b. Then each of these divided in the same incomplete fashion, forming a, a′, b and b′. Probably two of the four now attached divided, forming the six. There are alternative explanations, but this is the more probable one, since it means a maximum of three divisions each for four of the final six individuals, with two divisions each for the other two. The number of divisions is in agreement with the average for the culture.

In order to determine the validity of conclusions based on cultures aërated only at the beginning of experiments, several series of tests were carried out with cultures through which air was bubbled continuously. For convenience, only four H^+-ion concentrations were selected in each experiment. As controls for the continuously aërated cultures (in both light and darkness) duplicates were initially aërated and closed with cotton plugs.

Figure 7 represents the results of such a test. The continuous aëration does produce a somewhat greater rate of division, due to the large supply of carbon dioxide and oxygen, but the curves representing the rates under such conditions are similar in shape to those obtained from cultures only initially aërated. We may conclude from this that the effects of pH in cultures initially aërated and having access to the air are not qualitatively different from those in cultures continuously in equilibrium with the air. The results here are, however, based on experiments lasting for only forty-eight hours. Over longer periods of time, the changes in standing cultures might tend to make the pH-division-rate curve approach the form of that found for sealed cultures.

Observations on aërated cultures furnish little or no evidence for a pronounced optimum for the rate of division. This rate is fairly uniform over an extremely wide range of H^+-ion concentrations. A slight increase between pH 6 and 7 suggests an optimum there, but this is not nearly so apparent as that associated with photosynthesis alone.

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[3] All multiple organisms were counted as if all divisions begun had been completed. Inasmuch as these divisions are probably never completed, but followed by death, this method eliminates part of the true depression of division-rate at low pH.

All types of experiments consistently give us three explanations for the depression of division rate at low pH, exclusive of that associated with fatal injury to the organisms. (1) Etiolation, (2) production of multiple monsters which subsequently die, and (3) a photodynamic effect, all probably contribute to this depression. In addition, both high and low pH values are fatal—the limits found being pH 2.3 and 11.0 +. (*Euglena gracilis* will grow at pH 9.6, but its average rate of division is less than once in three days. No evidence of special effects at high pH has been obtained.)

It may be thought that the form of movement commonly observed in *Euglena,* and called variously "euglenoid movement" or "*Metabolie,*" may be induced by particular concentrations of hydrogen ions. In 1925, while working with *Euglena gracilis,* I found that organisms transferred from an acid medium to one distinctly more alkaline, or *vice versa,* began to go through this type of rhythmical contraction, and did not return to the normal swimming form for some time. The response is quite evidently associated with a change of environment, rather than with the nature of the new environment. It seems to the writer that the response to pH indicates that "euglenoid movement" is a modified avoiding reaction. It is certainly not a form of locomotion, for as a means of progress from place to place it is little, if at all, more efficient than Brownian movement.

Summary

1. The alleged tolerance of high concentrations of citric acid by *Euglena gracilis* Klebs is associated with H^+-ion concentration. This species of *Euglena* tolerates H^+-ion concentrations as high (or higher) when produced by hydrochloric, sulphuric or oxalic acids.

2. Even less acid solutions of valeric and salicylic acids cause death, however. This effect may be connected with the greater rates of penetration of these acids into living cells.

3. Increased growth of *Euglena gracilis* at high acidities is due to the elimination of bacterial or other competition at such pH values. It does not occur in a series of sterile cultures.

4. A sterile, pure-line, well-aërated culture of *Euglena gracilis,* in Bacto-peptone medium, showed little difference in division-rate between pH 7.7 and 4.5 in the light, and between pH 7.7 and 3.0 in darkness. An only slightly greater rate of division at about pH 6.7 suggests that a true optimum may exist at that point.

The absolute limits of life proved to be approximately pH 2.3 and 11.0 +.

5. There is a marked optimum pH for photosynthesis in this *Euglena*. Sealed cultures in the light invariably developed maximum growth at pH 6.5 to 6.8. Comparison with aërated cultures in darkness shows that the difference between growth of sealed cultures in light and in darkness is actually not due to the products of photosynthesis in the former, but to the use of the oxygen produced during photosynthesis in the oxidation of organic foods in the medium.

6. Quantitatively, enough oxygen is produced at pH 6.7, in photosynthesis by this *Euglena* to maintain its own reproduction at maximum or nearly maximum rate. *Euglena* may, therefore, be a significant element of the carbon cycle where it occurs in nature.

Under conditions of nearly complete anaërobosis in darkness it does not grow, but encysts, and subsequently dies.

7. In cultures of about pH 3.5 or lower, many individuals fail to complete normal division, remaining attached together by the surface at the posterior tip (the last part to divide in euglenoid fission). Two, three, four and even as many as six individuals have been found attached together, the higher numbers of individuals forming rosettes. This condition depends on pH, as such, occurring equally in light and darkness, in sealed and aërated cultures. Furthermore, it is apparently only a surface effect, as, although occasionally other types of abnormalities appear, in the type here described there is no connection between the cytoplasm of one individual and that of another.

8. More or less complete etiolation occurs, even under continuous illumination, below pH 5 ±. This is not permanent, the chlorophyl reappearing after the organisms are returned to a medium of lower H^+-ion concentration.

9. In cultures of low pH (below about pH 3.5) a photodynamic effect is present. This is far more marked with green than with etiolated individuals, which suggests that the contained chlorophyl, or a derivative of it, is the photodynamic compound involved.

10. Encystment does not occur as an effect of pH anywhere in the range studied, pH 8.5 to 2.4.

11. The so-called "euglenoid movement" is not induced by any particular pH, but may be brought about by transferring the organisms from the culture in which they have been living to one in which the pH is markedly different. This type of movement should not be considered a form of locomotion, but, rather, a modified avoiding reaction.

BIBLIOGRAPHY

Allee, W. C., 1930. Concerning Community Studies. *Ecology*, **11**: 621.

Blackman, F. F., 1905. Optima and Limiting Factors. *Ann. of Bot.*, **19**: 281.

Bresslau, E., 1926. Die Bedeutung der Wasserstoffionenkonzentration für die Hydrobiologie. *Verh. d. Int. Verein. f. theoret. u. angewandte Limnologie.*, **3**: 56.

Chalkley, H. W., 1930. On the Relation between the Resistance to Heat and the Mechanism of Death in Paramecium. *Physiol. Zoöl.*, **3**: 425.

Darby, H. H., 1929. The Effect of the Hydrogen Ion Concentration on the Sequence of Protozoan Forms. *Arch. f. Protist.*, **65**: 1.

Darby, H. H., 1930. The Experimental Production of Life Cycles in Ciliates. *Jour. Exper. Biol.*, **7**: 132.

Hargitt, G. T., and W. W. Fray, 1917. The Growth of Paramecium in Pure Cultures of Bacteria. *Jour. Exper. Zoöl.*, **22**: 421.

Harvey, E. N., 1928. Photosynthesis in Absence of Oxygen. *Plant Physiol.*, **3**: 85.

Hausmann, W., 1909. Die photodynamische Wirkung des Chlorophylls und ihre Beziehung zur photosynthetischen Assimilation des Pflanzen. *Jahrb. f. wiss. Bot.*, **46**: 599.

Jahn, T. L., 1929. Studies on the Physiology of the Euglenoid Flagellates. I. The Relation of the Density of Population to the Growth Rate of Euglena. *Biol. Bull.*, **57**: 81.

Klebs, G., 1883. Über die Organisation einiger Flagellatengruppen und ihre Beziehungen zu Algen und Infusorien. *Untersuch. aus d. bot. Inst. zu Tübingen.*, **1**: 233.

Koffmann, M., 1924. Über die Bedeutung der Wasserstoffionenkonzentration für die Encystierung bei einigen Ciliatenarten. *Arch. f. mikr. Anat.*, **103**: 168.

Kostir, W. J., 1921. The Comparative Resistance of Different Species of Euglenidæ to Citric Acid. *Ohio Jour. Science*, **21**: 267.

Loomis, A. L., E. N. Harvey and C. MacRae, 1930. The Intrinsic Rhythm of the Turtle's Heart Studied with a New Type of Chronograph, Together with the Effects of Some Drugs and Hormones. *Jour. Gen. Physiol.*, **14**: 105.

Parpart, A. K., 1928. The Bacteriological Sterilization of Paramecium. *Biol. Bull.*, **55**: 113.

Philpott, C. H., 1928. Growth of Paramecia in Pure Cultures of Pathogenic Bacteria and in the Presence of Soluble Products of Such Bacteria. *Jour. Morph. and Physiol.*, **46**: 85.

Powers, E. B., 1930. The Relation Between pH and Aquatic Animals. *Am. Nat.*, **64**: 342.

Robertson, T. B., 1922. Reproduction in Cell-Communities. *Jour. of Physiol.*, **56**: 404.

Skadowsky, S. N., 1926. Über die aktuelle Reaktion der Süsswasserbecken und ihre biologische Bedeutung. *Verh. d. Int. Verein. f. theoret. u. angewandte Limnologie*, **3**: 109.

Spoehr, H. A., 1926. Photosynthesis. New York.

Stiles, Walter, 1925. Photosynthesis. The Assimilation of Carbon by Green Plants. London.

Ternetz, Charlotte, 1912. Beiträge zur Morphologie und Physiologie der Euglena gracilis Klebs. *Jahrb. f. wiss. Bot.*, **51**: 435.

Wann, F. B., and E. F. Hopkins, 1927. Further Studies on Growth of Chlorella as Affected by Hydrogen-ion Concentration. *Bot. Gaz.*, **83**: 194.

Warburg, Otto, 1919. Über die Geschwindigkeit der photochemischen Kohlensäurezersetzung in lebenden Zellen. *Biochem. Zeitschr.*, **100**: 230.

Zumstein, Hans, 1899. Zur Morphologie und Physiologie der Euglena gracilis Klebs. *Jahrb. f. wiss. Bot.*, **34**: 149.

THE RESPIRATORY FUNCTION OF THE BLOOD OF URECHIS CAUPO

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Urechis caupo is an echiuroid worm inhabiting sandy mud flats in the estuaries of the coast of California. Its characters and habits have been described by Fisher and MacGinitie (1928). Because of its large size, the simple nature of its circulatory and respiratory systems, and the fact that its cœlomic fluid is voluminous and contains abundant red blood corpuscles, it affords unusually suitable material for the study of respiratory problems. The present paper contains a description of those properties of the blood of *Urechis* which are of importance in respiration, together with certain observations designed to evaluate the significance of these properties.

The material used for these studies was collected in the Elkhorn Slough, a tributary of Monterey Bay. The authors wish to express their indebtedness to Dr. MacGinitie for assistance in procuring the animals and to Professor Fisher for the many courtesies received while they were at the Hopkins Marine Station.

I. THE BLOOD OF URECHIS

Fisher and MacGinitie (1928) state that the cœlom is filled with bright red blood, the pigment being lodged in subcircular cells, about 0.025 mm. in diameter, which readily distort when crowded. There are also very numerous amœboid cells which when aggregated are yellow in color. We have found the color of the blood to vary, being frequently of a dull brownish-red color; less often, and particularly in smaller specimens, of a bright scarlet resembling the blood of vertebrates. A volume of 15 or 20 cc. may be secured from a single specimen.

The Blood

The plasma does not clot, and when separated from the cells is a pale yellow color; not infrequently it may be tinged with the corpuscular pigment. Under microscopic examination the cytoplasm of the corpuscles appears yellow and is seen to be filled with small, highly refractive granules. In addition, there are many granules of a brown pigment in the corpuscles of some specimens. The occurrence of this pigment is variable and it may be nearly lacking in those specimens whose blood appears scarlet rather than brownish-red. In the center of the corpuscle is a clearer area, which, on staining, proves to be a small nucleus. The cœlomic cells of *Urechis chelensis* described by Seitz (1907) are apparently similar, being nucleated and containing yellow pigment granules (in preserved material).

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[1] Fellow of the C. R. B. Educational Foundation.

TABLE I

Spectrometric Data of Urechis Hemoglobin

Wave Length	Oxyhemoglobin	Reduced Hemoglobin	Wave Length	Oxyhemoglobin	Reduced Hemoglobin
mμ	*E*	*E*	*mμ*	*E*	*E*
450.4	0.394	0.495	566.6	0.221	0.299
460.5	0.280	0.225	567.6	0.223	
470.6	0.226	0.169	569.7	0.245	0.273
480.7	0.189	0.160	571.7	0.285	
490.8	0.179	0.164	573.7	0.297	
500.9	0.174	0.176	575.7	0.309	
511.0	0.172	0.197	576.7	—	0.238
516.1	0.180		577.8	0.310	
521.1	0.192	0.218	578.9	0.297	
523.2	0.206		581.8	0.267	0.204
525.2	0.215		583.8	0.214	
527.2	0.231		585.8	0.168	
529.3	0.244		586.9	—	0.171
531.3	0.264	0.245	587.9	0.131	
533.3	0.276		589.9	0.102	
535.3	0.291		591.9	0.082	0.145
537.3	0.299		597.0	0.055	
539.3	0.306		602.0	0.042	0.096
541.4	0.312	0.277	612.1	0.032	0.073
543.4	0.310		622.2	0.025	0.064
545.4	0.292		632.4	0.022	0.059
546.4	—	0.298	642.5	0.024	0.056
547.4	0.276		652.6	0.009	0.049
549.5	0.255		662.7	0.016	0.046
551.5	0.243	0.313	672.8	0.004	0.038
555.5	0.214		682.9	0.008	0.036
556.5	—	0.313	693.0	0.003	0.028
559.6	0.197				
561.6	0.197	0.312			
563.6	0.202				
565.6	0.211				

The corpuscles appear to be surrounded by a strong membrane. On dilution of blood with distilled water, the cells swell but do not burst. Upon applying pressure to the coverslip when in this condition the membrane ruptures and the contents may be seen to flow out through a localized opening. The granules in the swollen corpuscles are in active Brownian movement, suggesting a fluid state of the interior of the cell.

In a one per cent saponin solution the cells swell, the granules remaining confined to the previous volume of the cells and appearing surrounded by a clear region. After a few minutes the membrane spontaneously ruptures and the granules flow out through the localized opening.

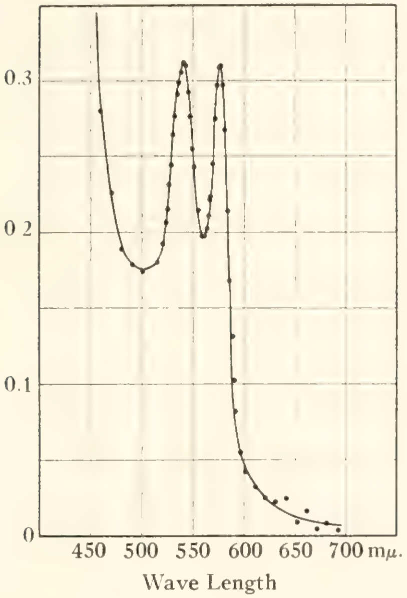

FIG. 1

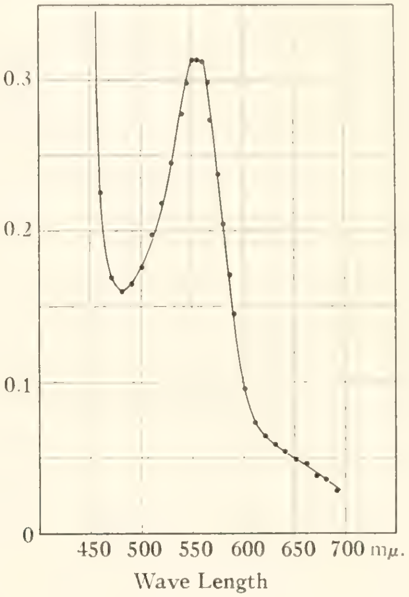

FIG. 2

FIG. 1. Absorption spectrum of oxygenated hemoglobin of *Urechis caupo.* Ordinates: extinction cœfficient of solution of unknown concentration.

FIG. 2. Absorption spectrum of reduced hemoglobin of *Urechis caupo.* Ordinates: extinction cœfficient of solution of same concentration as that shown in Fig. 1.

The Respiratory Pigment

That the corpuscles of *Urechis* contain hemoglobin is indicated by the spectroscopic examination of laked blood. Typical hemoglobin crystals may be obtained by allowing laked corpuscles to dry under a coverglass. For spectrophotometric examination a solution of hemoglobin was prepared by laking one cc. of corpuscles separated by centrifugation with 11 cc. of distilled water containing three drops of ether. To this solution was added 8 cc. of 4M ammonium sulfate brought to ca. pH 8 by the addition of ammonia. From this solution the corpuscular debris was filtered off and one volume of the filtrate diluted with four volumes of water. Filtration was repeated. The filtrate so obtained contained the original corpuscular content of hemoglobin diluted 1 : 100 and in the presence of 0.8 molar ammonium sulfate at a pH of approximately 8.

One sample of this solution was employed for measuring the absorption spectrum of oxyhemoglobin; another specimen was reduced by equilibration with hydrogen and used to obtain the spectrum of reduced hemoglobin. The solutions were perfectly clear. The measurements were made with a König-Martens spectrophotometer within five hours of the completion of the preparations. The length of the column of fluid was 3.3 cm. The extinction cœfficients of these solutions, estimated for one cm. length, are recorded in Table I and illustrated graphically in Figs. 1 and 2. In Table II are recorded the wave length of maximum density in the α and β bands and the wave length of minimum absorption between these bands as obtained by various workers with various hemoglobins.

TABLE II

Spectrometric Characteristics of Various Oxyhemoglobins

Species	Wave Length of Maximum Absorption		Wave Length of Minimal Absorption between α and β Bands	Ratio of Extinction Coefficients at Maximum β and Minimum between α and β	Observer
	α Band	β Band			
	$m\mu$	$m\mu$	$m\mu$		
Dog and horse. . .	575.6	540.4	558.1	>1.60	Hári (1917)
Dog.	575–577	539–542	560.*	1.63	Kennedy (1926–27)
Horse.	578.2	540.4	562.5*	1.58*	Vlès (1923)
Arenicola	576.0	540.0	560.*	1.53*	Vlès (1923)
Marphysa .	578.0	540.0			Vlès (1923)
Cucumaria	579.	542.7	558.		Van der Lingen and Hogben (1928)
Urechis. . . .	577.	542.	561.	1.58	

* Estimated from published data of observer.

These values for *Urechis* hemoglobin agree more closely on the whole with those found for the hemoglobin of the worm *Marphysa* and the holothurian *Cucumaria* and with Vlès' measurements of horse hemoglobin than with this author's data for *Arenicola*. The values for horse hemoglobin obtained by Vlès, from which he concluded that *Arenicola* hemoglobin differed from horse hemoglobin, do not agree with the values obtained by Hári and Foster for the mammalian pigment, which are very similar to Vlès' values for *Arenicola*. The shape of the absorption curve for *Urechis* does not agree exactly with the data recorded for mammalian oxyhemoglobin, particularly in the region about 510 mμ. Discrepancies in the shape of the curves may be attributed to the presence of methemoglobin in the solutions, as Hári has pointed out; a fact which makes the direct comparison of the curves difficult. The data leave no doubt that the pigment of the *Urechis* blood is a hemoglobin, but the spectrometric evidence regarding the specificity of the hemoglobin cannot safely be interpreted.

TABLE III

Cell Volume and Oxygen Capacity of Urechis Blood

Specimen	Volume Red Cells	Oxygen Content	Oxygen Combined	Oxygen Combined per 100 cc. Cells
	per cent	*volumes per cent*	*volumes per cent*	*cc.*
3	36.6	—	—	—
4	26.4	—	—	—
7	40.3	6.30	5.80	14.4
		7.22	6.72	16.7
8	37.6	5.83	5.33	14.2
		5.70	5.20	13.8
		5.77	5.22	13.9
9	18.3	2.85	2.35	12.8
		2.87	2.37	12.9
10	35.3	5.72	5.22	14.8
		5.53	5.03	14.2
12	23.8	4.64	4.14	17.4
		4.36	3.86	16.2
		4.24	3.74	15.7
13	19.5	3.89	3.39	17.4
		3.70	3.20	16.4
14	23.2	2.90	2.40	10.3
		2.83	2.33	10.0
		2.66	2.16	9.3
15	28.6	4.53	4.03	14.1
		4.30	3.80	13.3
16	32.0	4.54	4.04	12.6
		4.78	4.28	13.4
21	—	3.70	3.20	—
20	—	4.43	3.93	—
		4.50	4.00	—
23	—	4.09	3.59	—
	—	4.05	3.55	—

Hemoglobin appears to occur in the musculature of *Urechis*, particularly in that of the foregut, or crop. In this structure, which is in a thin muscular tube of a bright pink color, the spectrum of oxyhemoglobin can be beautifully demonstrated with the microspectroscope. If the preparation is covered with a coverglass, the spectrum soon changes to that of reduced hemoglobin, except near the edges, where the oxyhemoglobin bands persist. Because of the absence of capillaries in this preparation it should form very advantageous material for the study of the function and properties of muscle hemoglobin.

The Quantity of Corpuscles and Hemoglobin in the Blood

The red corpuscles occupy from 18 to 40 per cent of the total volume of the blood when separated with the hematocrit (Table III). A gray layer of rather variable volume containing sperm or eggs and other cells separates between the red cells and the plasma. The oxygen content of the blood equilibrated with air was determined with the Van Slyke constant volume blood gas apparatus, using one cc. samples, and varies between two and six volumes per cent. Special care was taken to stir the blood before sampling because of the rapid rate at which the large corpuscles settle out. A one per cent saponin solution was used as laking reagent. These values are recorded in Table III and may be compared with the values found for other worms and other invertebrates containing hemoglobin in Table IV.

TABLE IV

Oxygen Content of Blood of Worms and Other Invertebrates (equilibrated with air)

Species	Oxygen Content	Pigment	Occurrence	Observer
Urechis caupo......	2.66–7.22	Hemoglobin	in corpuscles	
Glycera siphonostoma	2.58–3.03	Hemoglobin	in corpuscles	Winterstein (1909)
Arenicola sp.........	5.70–8.70	Hemoglobin	in solution	Fox (1926) after Barcroft and Barcroft (1924)
Cardita sulcata......	1–2	Hemoglobin	in solution	Winterstein (1909)
Pectunculus violaceus	1–2	Hemoglobin	in solution	Winterstein (1909)
Spirographis........	8.10–10.0	Chlorocruorin	in solution	Fox (1926)
Siphunculus nudus...	ca. 2	Hemerythrin	in corpuscles	Winterstein (1909)

It is commonly believed that the inclusion of the respiratory pigments within corpuscles has made possible the superior oxygen capacity of the blood of vertebrates. This possibility does not appear to have been realized in the invertebrate stage of development, for *Arenicola* and

Spirographis, which carry their respiratory pigments in solution, have a greater oxygen content than *Urechis* and the other invertebrate forms in which oxygen is transported in blood corpuscles.

The concentration of hemoglobin in the corpuscles of *Urechis* appears to be much less than is the case in vertebrates. In Table III is recorded the estimated oxygen combined per 100 cc. of red corpuscles —allowance being made for 0.50 volumes per cent of oxygen assumed to be present in solution. The oxygen-combining power of the cells varies from about ten to seventeen volumes per cent. Drastich (1928) finds the following values for the hemoglobin content of the cells of vertebrates:—various mammals 29.5 to 34; various birds 29.54; *Rana esculenta* 24.85; carp 26.02 grams per 100 cc. corpuscles. Assuming the *Urechis* hemoglobin to have the same oxygen-combining power per unit weight as mammalian hemoglobin (one volume per cent oxygen capacity corresponding to 0.746 grams of hemoglobin per 100 cc.), *Urechis* corpuscles are estimated to contain 7.5 to 12.7 grams of hemoglobin per 100 cc. of cells. The *Urechis* corpuscle is then about one-third as effective in transporting oxygen as those of the vertebrates. It is to this fact rather than to a deficiency in the number of corpuscles that the low oxygen capacities of the blood are principally due.

TABLE V

Data on Equilibrium of Oxygen with Urechis Blood

Carbon Dioxide Pressure	Oxygen Pressure	Oxygen Content	Oxygen Dissolved	Oxygen Combined	Saturation
mm. Hg	*mm. Hg*	*volume per cent*	*volume per cent*	*volume per cent*	*per cent*
8.64	5.98	0.63	0.192	0.44	19.1
9.78	8.50	0.87	0.027	0.84	36.6
8.61	12.15	1.15	0.039	1.11	48.3
7.90	16.35	1.46	0.053	1.41	61.3
7.54	23.08	1.88	0.074	1.81	78.8
10.87	41.85	2.09	0.135	1.95	84.8
6.54	48.15	2.15	0.155	1.99	86.6
6.11	72.61	2.32	0.234	2.09	90.9
7.56	87.21	2.36	0.281	2.08	90.4
air	air	2.90	0.50	2.40	
		2.83	0.50	2.33	
		2.66	0.50	2.16	
				av. 2.30	100

Iron Content of Blood

Attempts to estimate the iron content of the blood by the method of Hall and Gray (1929) yielded rather discordant results. The values obtained were always of the order expected from the oxygen capacity of the samples.

The Equilibrium of Oxygen with the Blood

The oxygen dissociation curve of the whole blood has been determined using the Van Slyke constant volume apparatus for blood gas estimations and the Haldane analyzer for measuring the composition of the gas with which the blood has been equilibrated. Equilibration was carried out upon 3 cc. of blood enclosed in 250 cc. tonometers rotated for 20 minutes in a water bath at 19° C. Analyses were made immediately after equilibration in fear that the metabolism of the cells might alter the gaseous content were the samples allowed to stand. The carbon dioxide pressure was maintained approximating that obtaining in the blood *in vivo;* about seven millimeters. The data are recorded in Table V.

In estimating the combined oxygen from the oxygen capacity it is assumed that blood in equilibrium with air dissolves 0.5 volumes per cent oxygen, the solubility at lower oxygen pressures being proportional in accordance with Henry's law. The oxygen dissociation curve is plotted in Fig. 3.

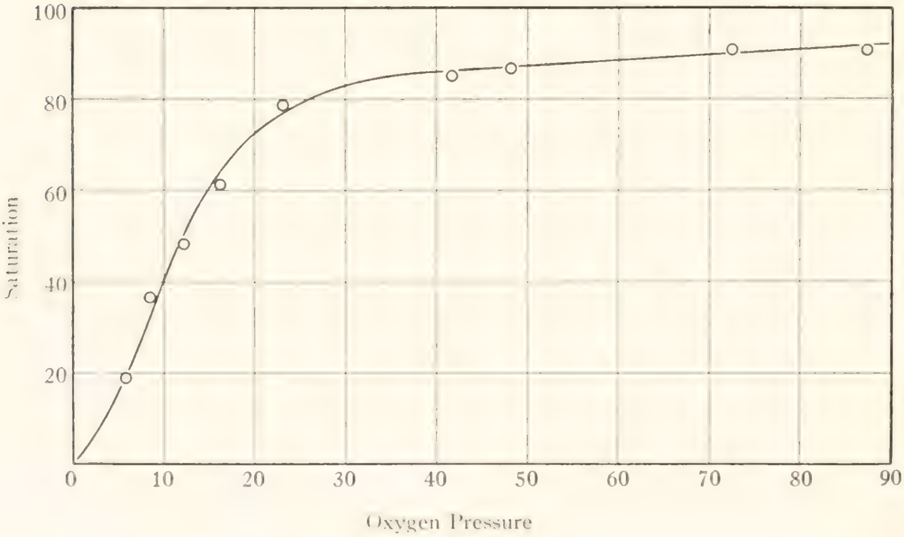

FIG. 3. Oxygen dissociation curve of blood of *Urechis caupo.* Temperature 19° C. For data see Table V. Ordinates: percentage of saturation; abscissæ: partial pressure of oxygen in mm. Hg.

The Effect of Carbon Dioxide upon the Oxygen Dissociation Curve

Samples of blood have been equilibrated with oxygen in the presence of carbon dioxide at pressures varying from 0.54 to 92 mm. Hg. The temperature of equilibration was 19° C. The results are recorded in Table VI. From this data, p_{50}, the oxygen pressure at which the blood would have been half saturated with oxygen has been calculated, assuming the curves to have the same shape as that drawn in Fig. 3.

TABLE VI

Data on Equilibrium of Blood with Oxygen at Various CO_2 *Pressures*

Specimens	Carbon Dioxide Pressure	Oxygen Pressure	Oxygen Content	Oxygen Dissolved	Oxygen Combined	Saturation	p_{50}
	mm. Hg	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*mm. Hg*
Urechis No. 15	0.54	10.40	1.96	0.03	1.93	49	10.6
	0.94	14.89	2.62	0.04	2.58	66	10.7
	8.60	7.10	2.16	0.02	2.14	55	6.3
	8.82	14.12	2.60	0.04	2.56	66	10.1
	19.60	8.40	2.25	0.12	2.23	57	7.2
	19.80	16.25	2.68	0.05	2.63	67	11.3
	29.40	12.55	2.20	0.04	2.16	55	11.2
	air	air	4.53	0.50	4.03	103.3	—
			4.30	0.50	3.80	97.4	—
Urechis No. 16	0.62	8.38	1.74	0.03	1.71	41.	9.9
	0.71	10.80	1.95	0.03	1.92	46.	11.7
	0.76	9.56	1.96	0.03	1.93	47.	10.1
	1.26	9.44	1.77	0.03	1.74	42.	11.1
	77.0	9.08	2.14	0.03	2.11	51.	9.0
	92.0	11.30	2.34	0.04	2.30	55.	10.1
	air	air	4.54	0.50	4.04	97.	—
			4.78	0.50	4.28	103.	—

These values, recorded in the last column of Table VI, make it appear that the affinity of the blood for oxygen is not influenced to a detectable degree by the pressure of carbon dioxide within the ranges of pressure examined. In this regard the blood of *Urechis* differs from that of most vertebrates and from that of *Arenicola.* In the latter form Barcroft and Barcroft (1924) found the typical effect of hydrogen ion concentration upon the oxygen dissociation curve. Recently Dill and Edwards (1931) have observed that in the blood of the elasmobranch, *Raia oscillata,* the effect of carbon dioxide upon the oxygen dissociation curve is absent or nearly so.

The Effect of Temperature upon the Oxygen Dissociation Curve

Oxygen dissociation curve data obtained from the same specimen of blood have been secured at two temperatures, 22° C. and 34.5° C. (Fig. 4). The carbon dioxide tension was about 12 mm. in both cases.

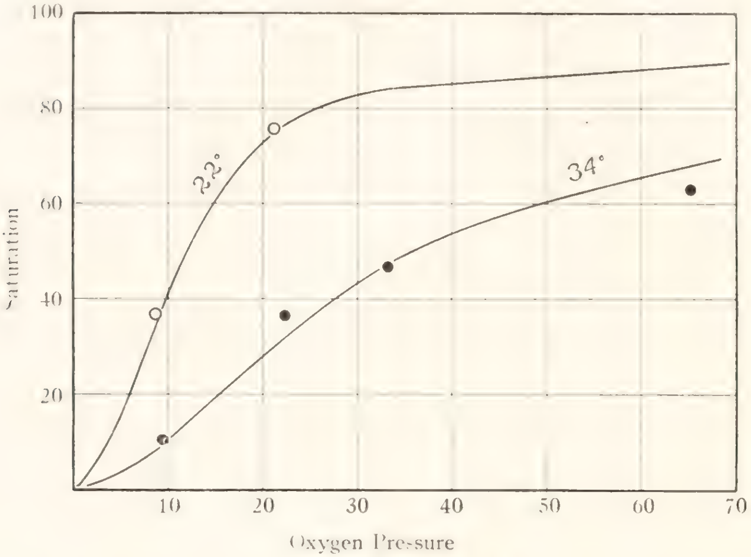

FIG. 4. Oxygen dissociation curves of blood of *Urechis caupo*, equilibrated at temperatures of 22° C. and 34° C. Ordinates: percentage of saturation; abscissae: partial pressure of oxygen in mm. Hg.

The curve drawn through the data obtained at 22° C. is identical with that in Fig. 2 obtained from another sample of blood at 19° C. At 34° C. the points lie well to the right. The data are insufficient to warrant any conclusion with regard to the shape of the curve at the higher temperature, but it is clear that the temperature effect upon the oxygen equilibrium is large and of the same direction and order observed in vertebrate hemoglobin (Brown and Hill, 1923; Macela and Seliškar, 1925).

The Equilibrium of Carbon Dioxide with the Blood

Table VII presents the data obtained by equilibrating *Urechis* blood against various mixtures of carbon dioxide in air at 18.5° C. The analyses were made with the Van Slyke apparatus and the Haldane analyser. The oxygen capacity of the blood employed corrected for dissolved oxygen was 3.9 volumes per cent.

In order to facilitate comparison of the *Urechis* blood with that of

TABLE VII

Data on Equilibrium of Carbon Dioxide with Urechis Blood

Carbon Dioxide Pressure	Carbon Dioxide Content	Carbon Dioxide Dissolved (H_2CO_3)	Carbon Dioxide Combined ($BHCO_3$)	$\log \frac{(BHCO_3)}{(H_2CO_3)}$
mm. Hg	*vol. per cent*	*vol. per cent*	*vol. per cent*	
0.9	3.32	0.09	3.23	1.597
3.3	6.13	0.35	5.78	1.217
7.22	8.90	0.76	8.14	1.029
12.40	11.00	1.30	9.70	0.873
22.0	14.15	2.31	11.84	0.710
47.2	19.15	4.96	14.19	0.456

other animals, and for the comparison of various experiments with this species it is convenient to relate the data to the logarithm of the ratio of combined ($BHCO_3$) to free (H_2CO_3) carbonic acid. This function changes approximately in proportion to the hydrogen ion concentration, which may be obtained by adding the appropriate pK value. Moreover, the total buffer value of the blood is also dependant upon this function. The quantity of carbon dioxide dissolved in the blood or present as H_2CO_3 (free carbonic acid) has been estimated assuming α, the number of cubic centimeters of CO_2 dissolved in one cubic centimeter at a pressure of 760 mm. Hg, to be 0.80. This value is slightly less than the value 0.827 given by Bohr (1897) for two per cent NaCl at 18° C.

The concentration of combined carbonic acid ($BHCO_3$) is obtained by subtracting the free carbonic acid (H_2CO_3) from the total carbonic acid. The estimated values of these quantities are included in the table.

The total buffer value of blood, β, is defined by the equation

$$\beta = \frac{-\Delta(BHCO_3)}{\Delta \log \frac{(BHCO_3)}{(H_2CO_3)}}$$

In Fig. 5 the values of ($BHCO_3$) are plotted against $\log \frac{(BHCO_3)}{(H_2CO_3)}$. Throughout a considerable range the points fall about a straight line, indicating as in the case of mammalian blood that the buffer value is constant. The value of β is given by the slope of this line and is 11 volumes per cent (or 0.49 milliequivalents per liter).

It will be shown subsequently that the plasma of *Urechis* possesses little or no buffer value. Is the total buffer value of the *Urechis* blood adequately accounted for by the quantity of hemoglobin in the corpuscles? In this specimen of blood the oxygen capacity was 3.9 volumes per cent. The buffer value per equivalent of hemoglobin is given by $\beta/3.9 = 2.82$. This value is intermediate between the buffer values of oxygenated and reduced hemoglobin as it occurs in the cells of the blood of man and of the crocodile, the extreme values being 2.4 for reduced crocodile blood at 29° and 3.47 for oxygenated blood of this species (Dill and Edwards, 1931). The concentration of hemoglobin appears sufficient to account for the total buffering effect of *Urechis* blood.

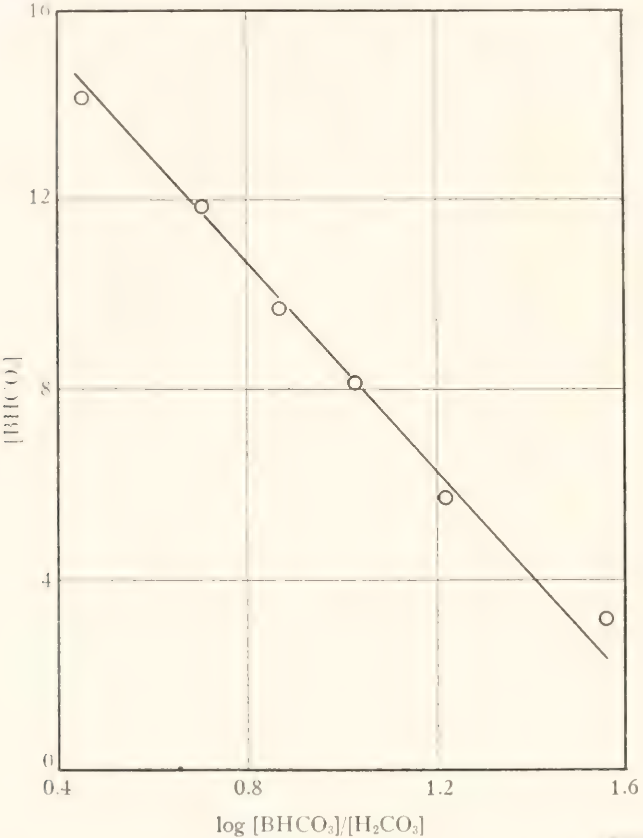

FIG. 5. Relation of combined carbonic acid ($BHCO_3$), to log $\frac{(BHCO_3)}{(H_2CO_3)}$ for blood of *Urechis caupo*. Temperature 19° C. For data see Table VII.

The effect of oxygenation and reduction upon the carbon dioxide-combining power of blood is considered to be directly related to the reciprocal effect of carbon dioxide (or hydrogen ion concentration) upon the oxygen dissociation constant of hemoglobin (Henderson, 1928. Chapter IV). We have shown in the case of *Urechis* that the latter is uninfluenced by the quantity of carbon dioxide present, and it is consequently interesting to inquire whether reduced blood possesses the same carbon dioxide-combining power as oxygenated blood. Dill and Edwards (1931) state that both effects are absent or nearly so in the blood of the skate, *Raia oscillata*.

As the effect is proportional to the concentration of hemoglobin, it may be expected to be small in any case. In order to facilitate the experiments, the blood of about ten animals was mixed and the corpuscles separated with the centrifuge. A small quantity of plasma was mixed with the corpuscles, yielding a solution containing 78 per cent red blood corpuscles and having an oxygen capacity of 10.7 volumes per cent. Were the *Urechis* hemoglobin similar to mammalian hemoglobin the reduced solution should combine four or five volumes per cent more carbon dioxide than the oxygenated serum when log $\frac{(BHCO_3)}{(H_2CO_3)}$ is 0.8. Table VIII shows the result of equilibrating samples of this concentrated blood with air and with hydrogen containing about twenty-two mm. pressure of carbon dioxide. The quantity of oxygen found in the tonometers used for the " reduced " samples would not oxygenate more than ten per cent of the hemoglobin. Disregarding the first experiment of the series, the combined carbon dioxide is the same within the limits of experimental error in both series.[2] Certainly the phenomenon does not occur with the magnitude commonly observed in the blood of the higher vertebrates, and as in the case of the skate, it may be concluded that the reciprocal effects of oxygen and carbon dioxide upon the equilibrium of *Urechis* hemoglobin with these gases are absent or nearly so.

TABLE VIII

Data on Carbon Dioxide Equilibrium in Oxygenated and Reduced Blood

Oxygen Pressure	Carbon Dioxide Pressure	Carbon Dioxide Combined
mm. Hg	*mm. Hg*	*vol. per cent*
air	21.48	13.42
air	21.50	11.50
air	19.85	10.90
air	23.90	11.21
3.54	23.62	11.85
3.70	23.65	11.50

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[2] A second experiment in which the corpuscles were not so highly concentrated and in which the measurements did not agree so closely yielded higher CO_2 values for the oxygenated than for the reduced samples.

The distribution of carbon dioxide between the cells and plasma is of particular interest as *Urechis* is the most primitive type of animal in which the respiratory properties of the corpuscles have been studied. The red blood corpuscle of *Urechis* is a much more "typical" cell than are those of the vertebrates and one looks for properties which contrast it with these more highly specialized erythrocytes.

TABLE IX

Data on Distribution of Carbon Dioxide between Corpuscles and Plasma

Specimen No.	19	22	24	25	26
Temperature of equilibration—° C.	19.5	19.0	20	19	19
Volume of corpuscles—*per cent*	23.0	34.5	23	34.7	31.7
Low Pressure Experiment					
CO_2 pressure of equilibration—*mm. Hg.*	8.0	12.95	10.50	10.20	9.31
Whole blood-CO_2 content—*vol. per cent*	8.82	14.20	11.40	11.18	10.80
True plasma-CO_2 content—*vol. per cent*	7.09	14.15	11.30	10.95	11.45
Separated serum					
CO_2 pressure of equilibration—*mm. Hg.*	53.6	68.4		54.7	47.0
CO_2 content at this pressure—*vol. per cent*	11.74	19.30		16.54	15.40
High Pressure Experiment					
CO_2 pressure of equilibration—*mm. Hg.*	62.5	62.2		49.5	46.7
Whole blood-CO_2 content—*vol. per cent*	20.7	23.5		19.4	19.56
True plasma-CO_2 content—*vol. per cent*	18.9	20.4		17.12	18.82

Several experiments have been made in order to elucidate the respective parts which corpuscles and plasma play in the transport of carbon dioxide, and to determine the extent to which there is an exchange of material between cells and plasma. The procedure has been to equilibrate blood with carbon dioxide at a pressure comparable to that existing *in vivo*. With a part of this solution duplicate determinations were made of the carbon dioxide content of the *whole blood*. The remainder was centrifuged under oil in stoppered tubes. A portion of the plasma so separated was analyzed for the carbon dioxide content of the *true plasma, i.e.,* the plasma in equilibrium with corpuscles at the original carbon dioxide tension. From these measurements together with a measurement of the fraction of the whole blood occupied by the corpuscles, made by hematocrit, the ratio of carbon dioxide concentration in corpuscles and plasma could be calculated. The remainder of the plasma was then equilibrated with a relatively high CO_2 tension in order to give an idea of the buffer action of the *separated plasma*. The foregoing measurements, which are designated as the "low pressure experiment" in the tables, were performed in the mornings on which the blood was drawn. In the afternoon the "high pressure experiment" was carried out. A portion of the whole blood was now equilibrated at a relatively high CO_2 pressure and its CO_2 content and that of the true plasma determined. The data of the experiments are given in Table IX and certain calculations based on these data appear in Table X. Referring to the latter, several definite conclusions may be drawn. In other regards the results of the experiments are at variance and the interesting question emerges as to whether the apparent variability in behavior may be due to the properties of the relatively unspecialized cell which serves as erythrocyte in *Urechis.*

TABLE X

Certain Indices of the Distribution of Carbon Dioxide between Corpuscles and Plasma

Specimen No.	19	22	24	25	26
Ratio of CO_2 concentration between corpuscles and plasma					
Low pressure experiments	(2.05)	1.01	1.04	1.03	0.82
High pressure experiments	1.43	1.43	—	1.38	1.29
Change in CO_2 content per unit change in CO_2 pressure					
Separated serum—*vol. per cent per mm. pressure*	0.102	0.093	—	0.125	0.105
True serum—*vol. per cent per mm. pressure.*	(0.217)	0.127	—	0.156	0.197
Whole blood—*vol. per cent per mm. pressure*	0.218	0.189	—	0.208	0.234
Total buffer value of blood; β	9.6	7.3		7.6	9.2
Volume of corpuscles—*per cent*	23.0	34.5	23.00	34.7	31.7

The ratio of the CO_2 concentration of whole blood to that in the true plasma is approximately 1.0 in the low pressure experiments. The discordant value in Experiment No. 19 is probably to be attributed to experimental error. This means that CO_2 is about equally distributed between corpuscles and plasma under pressure conditions such as occur in the blood of the worm. The effects of the Donnan equilibrium and the corrections for the volume occupied by solutes in the corpuscles and plasma are neglected and would probably be too small to be significant in measurements as inaccurate as those employed.

The ratio of carbon dioxide concentration between cells and plasma in the high pressure experiments is uniformly greater than one. This result indicates that the principal buffer substances occur within the corpuscle and that the exchange of materials between corpuscles and plasma (the chloride shift) which enables the corpuscles to contribute to the buffer action of the plasma in the higher vertebrates is restricted in the case of the *Urechis* blood corpuscle. It has been shown above that the hemoglobin concentration is sufficient to account for the total buffer action of *Urechis* blood. The apparent restriction in the exchange of electrolytes between corpuscles and plasma is perhaps to be related to the tough membrane which may be observed to surround the erythrocyte of *Urechis*.

The relative part played by corpuscles and plasma in *Urechis* blood is further expressed by the estimation of the change in CO_2 content of the components per unit change in CO_2 pressure in passing from the low to the high pressure stages of the experiment. This method of expressing the results is somewhat arbitrary, as the relation is not strictly comparable for varying ranges of pressure. However, for the present purpose of comparing data made at two similar pressures considerably separated, it is convenient.

The increase in CO_2 content with increase of pressure in the case of the *plasma separated at low tensions* is fairly uniform and has an average value of 0.106 volumes per cent per millimeter pressure. This is almost exactly the rate of increase which would be due to the solution of carbon dioxide in the plasma if the absorption cœfficient is 0.80 as assumed above. Parsons and Parsons (1923) publish some measurements of the carbon dioxide content of sea water at various CO_2 pressures from which it appears that the rate of increase is 0.109 volumes per cent per millimeter pressure. It is concluded that the plasma of *Urechis* contains at most a negligible quantity of buffer material, the increase in CO_2 content being adequately accounted for by the solubility of carbon dioxide.

Turning to the whole blood, the increase in CO_2 content with change in pressure is reasonably concordant in the various experiments and yields values about twice as great as in the case of the separated plasma. Between the pressures examined the gain in bound CO_2 is about equal to the gain in CO_2 dissolved.[3] This is a further expression of the fact that the corpuscular content is responsible for the buffer action of the blood. The buffer values for the whole blood recorded in Table X are slightly less than that estimated from the experiment recorded in Fig. 5. The variation in buffer value in different samples of blood does not appear to be closely correlated with the volume of cells in the samples. Presumably the variation in hemoglobin concentration in the cells from different specimens indicated in Table III is sufficient to destroy the expected correlation.

The true plasma shows a gain in CO_2 content with increasing CO_2 pressure which is variable but always less than the corresponding gain for whole blood and always greater than the gain shown by plasma separated at low pressures. The latter fact indicates that with increasing CO_2 pressure some exchange of material between corpuscles and plasma takes place which increases the ability of the plasma to take up carbon dioxide. This is presumably a " chloride shift " such as occurs in mammalian blood. With the exception of Experiment No. 19 the true plasma always gains less carbon dioxide than does whole blood. This is a further expression of the fact, brought out in the consideration of the ratio of carbon dioxide concentration in cells and plasma, that the exchange of materials affecting buffer action is limited. In Experiment No. 19 the high value of the carbon dioxide uptake of the true plasma is probably due to the experimental error which caused the ratio of carbon dioxide in cells and plasma to appear abnormal.

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[3] Over a shorter range the gain in bound CO_2 would be relatively greater than this because of the "shape" of the carbon dioxide-combining curve.

II. Physiological Observations

The Oxygen Content of the Blood in vivo

Samples of blood were drawn from worms lying in a pan of fresh sea water by inserting into the cœlomic cavity a hypodermic needle attached to a graduated one cc. pipette. The blood flowed into the pipette from its own pressure and was transferred directly into the Van Slyke apparatus for analysis.[4] Immediately following a larger sample of blood was drawn, equilibrated with air for 20 minutes and analyzed, thus yielding a measure of the oxygen capacity of the blood. Table XI contains the results of three such experiments. The figures are not corrected for dissolved oxygen. In Experiment No. 9 the oxygen content of the blood *in vivo* is equal to that of blood saturated with air.

Table XI

The Oxygen Content of Urechis Blood in vivo

Experiment No.	In vivo		Saturated with Air	
	Temperature	Oxygen Content	Temperature	Oxygen Content
	° C.	*volumes per cent*	*° C.*	*volumes per cent*
9	15	2.87	18	2.85
			18	2.87
20	18.5	3.45	19	3.70
21	18.5	4.11	19.5	4.43
			19.5	4.50

---

[4] The pressure existing in the cœlomic fluid may be about sixteen grams per cm.^{2}, for on one occasion in drawing blood from the cœlomic cavity the blood rose in the pipette to a vertical distance of 16 cm. and oscillated about this level as the result of the muscular contractions of the body wall.

In the other two experiments the oxygen content is slightly less than the oxygen capacity. The result indicates that the pressure of oxygen in the blood may be considerably less than that of air. The diminished oxygen content in the blood *in vivo* is largely due to the smaller amount dissolved rather than to incomplete oxygenation of the hemoglobin. Thus, in the case of Experiment No. 21, if we assume the oxygen pressure to be 75 mm., the hemoglobin would be 97 per cent saturated. The combined oxygen would amount to 3.85 volumes per cent if the total combining capacity be taken as 3.96 volumes per cent. The dissolved oxygen would be 0.25 volumes per cent, making the total content of the blood 4.10 volumes per cent as observed. It is concluded that the hemoglobin of *Urechis* is almost completely saturated when an abundant supply of oxygenated water is available for respiratory purposes, but that the pressure of oxygen in the blood may be considerably lower than that existing in the water.

TABLE XII

The Carbon Dioxide Content of Urechis Blood in vivo

Specimen No.	In vivo		In vitro		
	Temperature	CO_2 Content	O_2 Content†	CO_2 Pressure	CO_2 Content
	°C.	*vol. per cent*	*vol. per cent*	*mm. Hg*	*vol. per cent*
10	15	7.12	5.62		
11	15.5	8.12	4.65	10.4* 36.0*	10.67 18.95
12	16.5	8.79	4.4	See Table VII	

* Equilibrated at 18° C.

† Equilibrated with air. Not corrected for dissolved oxygen.

The Carbon Dioxide Content of the Blood in vivo

Table XII contains data on the carbon dioxide content of the blood *in vivo* obtained in a manner similar to that of the oxygen capacity. The measurements indicate a normal carbon dioxide content of between seven and nine volumes per cent. Data for the equilibrium of carbon dioxide with the blood used in Experiment No. 12 are recorded in Table VII and Fig. 5. Similar data for two points on the CO_2 dissociation curve of the blood used in Experiment No. 11 are included in Table XII. These data agree closely with those in Table VII. From Table VII it appears that at the CO_2 content observed *in vivo*, 8.79 volumes per cent, the pressure of carbon dioxide would be approximately 7.2 millimeters.

The pH Value of Urechis Blood in vivo

The pH value of blood plasma is given by the equation

$$pH = pK' + \log \frac{(BHCO_3)}{(H_2CO_3)},$$

in which pK′ is a constant dependant upon the properties of the corpuscles and plasma. The value of pK′ for *Urechis* blood is unknown, but it cannot differ greatly from 6.1 when the corrections for temperature (Warburg, 1922), ionic strength (Hastings and Sendroy, 1925) and corpuscular content (Van Slyke, Hastings, Murray and Sendroy, 1925) are taken into account. Since the value of $\log \frac{(BHCO_3)}{(H_2CO_3)}$ for *Urechis* blood at 7.22 mm. is about one, the pH value of the blood plasma *in vivo* must be close to 7.1. The blood is therefore somewhat more acid than human blood and much more acid than sea water. The value of $\log \frac{(BHCO_3)}{(H_2CO_3)}$ indicates that one eleventh of the carbon dioxide of the blood is in solution, the remainder being bound as bicarbonate.

The Exchange of Gas between Blood and Sea Water

Urechis lives in a permanent burrow in flats which are occasionally exposed at low tide. The burrows are U-shaped, having two openings. Water is circulated through the burrow by means of peristaltic contractions of the body wall, which force the fluid backward between the worm and the wall of the tube. The flow thus established serves both for respiration and to bring the animal its food supply. The worms may be kept for long periods in the laboratory confined in artificial burrows constructed of glass tubing, and under these conditions the volume of water circulated and the changes in its gaseous content may be measured. The respiratory and feeding reactions of animals so confined are fully described by Fisher and MacGinitie (1928). They consider that respiration is principally effected by means of water pumped into the hind-gut, through the activity of the muscular cloaca. The structure of the hind-gut as well as the active rhythm through which it is ventilated certainly support this view.

The hind-gut is a large sack extending the length of the body and occupying the greater part of the cœlomic cavity. Its wall is smooth and so thin as to be quite translucent, resembling in this regard a mesenteric membrane. The wall of the hind-gut is bathed directly by the blood, there being no blood vessels (Fisher and MacGinitie, 1928). The peristaltic movements of the body wall must produce some circulation in the blood. Rhythmic contractions of the hind-gut wall appear to be important in bringing the blood into contact with the hind-gut wall as well as in mixing the water in the hind-gut. If the worm is examined against the light one may see the outlines of the hind-gut, which appears as a relatively transparent region. It may be observed that the hind-gut is the seat of antiperistaltic contractions which sweep over it in the form of deep annular constrictions into which the blood is drawn and carried along. Compared to this effective mechanism the thick, muscular, cuticulated body wall must absorb a relatively small amount of oxygen.

The water within the hind-gut is renewed by a somewhat irregular rhythm. Fresh water is drawn in by a series of from one to upward of thirty small inhalations usually uninterrupted by exhalation. It is then discharged by means of a single exhalation, frequently followed by a period of rest. Fisher and MacGinitie record periods of inhalation lasting from twenty-five to ninety seconds and expirations consuming from ten to fifty seconds.

Samples of hind-gut water have been collected at the moment of expiration. The worm is watched through the glass wall of the aquarium until it begins to discharge the water in a vigorous stream which may be easily observed. At that moment the worm is taken out of the aquarium and the anal end thrust tightly into a funnel terminating under oil in a suitable glass-stoppered bottle. The worm continues to discharge the hind-gut water, which is collected under the oil. From 25 to 35 cc. of water may be obtained at a single discharge. Several discharges were combined to yield material for oxygen analysis by the Winkler method. Carbon dioxide content has been determined with the Van Slyke apparatus. Table XIII contains the results of a number of such determinations, together with control measurements made upon the sea water of the aquarium.

TABLE XIII

Oxygen and Carbon Dioxide Content of Hind-gut Water

Oxygen

Hind-gut water	0.37,	0.37,	0.29,	0.36,	0.36,	0.37	vol. per cent
Aquarium water	0.56,	0.56					vol. per cent

Carbon Dioxide

Hind-gut water	5.26,	5.11	vol. per cent
Aquarium water	4.77,	4.79	vol. per cent

The oxygen content of the expired water is about two-thirds that of the sea water. The partial pressure of oxygen in the hind-gut water is thus about one hundred millimeters and is quite sufficient to account for the high degree of saturation found in the blood *in vivo*.

The carbon dioxide measurements may be evaluated by means of determinations made by Parsons and Parsons (1923) of the carbon dioxide content of sea water from the Naples aquarium at various pressures. They found at 0.8 mm. CO_2 pressure a content of 4.7 volumes per cent which agrees closely with the values found in our aquaria. Interpolating from their data the carbon dioxide pressure of the expired water corresponds to 4.6 and 6.0 mm. in the two samples examined. Since the partial pressure of CO_2 in the blood is about seven millimeters, a gradient of pressure of about two millimeters occurs across the hind-gut wall.

From the foregoing experiments certain deductions may be drawn relative to the volume of water necessary to "ventilate" the hind-gut. Dr. V. E. Hall, who has been engaged in a study of the respiratory and feeding reactions of *Urechis,* has kindly supplied data concerning the volume of water pumped by *Urechis* through artificial burrows made from glass tubing, and the rate of oxygen consumption of the worms. The average rate of oxygen consumption of two medium-sized *Urechis* was about 0.013 cc. per minute. The amount of water pumped when the worms were not engaged in feeding was about 11 cc. per minute; when feeding, it was about 29 cc. per minute. There is required 2.3 cc. of sea water containing 0.56 volumes per cent oxygen to yield the 0.013 cc. consumed in one minute. When the water is expired from the hind-gut only one-third of the oxygen dissolved in it has been consumed. Consequently 6.9 cc. of water must ventilate the gut each minute. This is about half the amount pumped through the burrows when feeding is not going on. Feeding worms pump about four times the required volume of water, but under these circumstances the water is serving to bring food to the animal as well as for respiration. The size of the animals and their activity are variable and consequently these estimations cannot be very exact. They show, however, that the respiratory activity of the animal is rather nicely adjusted to the metabolic requirements.

The Function of the Hemoglobin of Urechis

The data in Table XI show that the hemoglobin of *Urechis* is almost completely saturated when an abundant supply of aërated water is available to the animals. The preceding considerations indicate that the mechanisms for bringing fresh water into contact with the respiratory surface of the hind-gut operate with a fair margin of safety at each step. Under ordinary conditions it appears that the oxygen bound to the hemoglobin is not utilized and that the oxygen dissolved in the plasma is sufficient for the metabolic requirements. *Urechis* must be added to the list of animals, including *Planorbis* (Leitch, 1916) and *Lumbricus* (Jordan and Schwarz, 1920), in which the hemoglobin does not appear to function if the oxygen supply is adequate.

Light is thrown on the possible value of the hemoglobin to the worms by considering the rate at which oxygen " circulates " through the blood. The problem is somewhat less definite in *Urechis* than in the vertebrates because there are no blood vessels and the ordinary conceptions of arterial, capillary and venous blood do not apply. If we consider 20 cc. to be the blood volume of *Urechis* and four volumes per cent to represent the oxygen capacity, then the oxygen content of the total blood is 0.8 cubic centimeters. Taking the rate of oxygen consumption to be 0.013 cc. per minute, it follows that only one-sixtieth of the oxygen content of the blood is used (and need be replaced) per minute. It is clear from this why the *Urechis* blood is almost completely saturated *in vivo*. It also follows that those properties which assist mammalian blood to give off or take up oxygen and carbon dioxide rapidly during its passage through the capillaries (the reciprocal action of oxygen and carbon dioxide on the equilibrium of these gases with hemoglobin and the transfer of buffer action from cells to plasma) may be dispensed with in *Urechis* blood.

The blood of *Urechis* appears from the foregoing observations to contain a store of oxygen sufficient to last the animal one hour. In addition the hind-gut water itself, having a volume of about thirty cc., contains some 0.11 cc. oxygen. This would serve to supply the metabolic requirement for not more than 8.5 minutes. The total oxygen within the animal consequently is enough to last about seventy minutes.

Consider what would happen if the blood contained no hemoglobin. In it the oxygen concentration would be no greater than in the hind-gut water, say 0.37 volumes per cent. The total volume of oxygen in 20 cc. of blood would be .074 cubic centimeters. At a metabolic rate of 0.013 cc. per minute this would last the animal 5.7 minutes. Adding to this the time which the oxygen in the hind-gut would serve, the total oxygen within an animal without hemoglobin would last about fourteen minutes.

The hemoglobin of *Urechis* consequently extends the period during which the respiratory exchange might be interrupted without depriving the animal of oxygen about five-fold, or for about fifty-five minutes. This is not long enough to carry the animal over the period of a low tide, when the burrows are exposed. It is sufficient to be useful during the " rest periods " which occur after a more or less prolonged period of feeding. According to Fisher and MacGinitie (1928), these rest periods are of two sorts: (1) intermittent periods of from 4.5 to 8.5 minutes separated by about 1.5-minute intervals, during which water is expelled from the respiratory chamber and a new supply taken, (2) a continuous rest of an hour or more during which respiration ceases (or at least is so reduced as to be imperceptible) and no movement of any kind takes place.

The Oxygen Supply When the Tide is Out

On the California coast the tides follow a rhythm in which alternate tides are of unequal height. The low course tides are more nearly equal and in the estuary where *Urechis* was found the flats are not uncovered. During the high course tides the flats are uncovered once a day for a period of six or more hours. During the greatest spring tides the estuary sometimes empties so completely during the lower ebb tides that it does not fill during the succeeding flood tide and in consequence the flats may be bare for 18 hours.

During the period when the tide is out there is available for the worms not only the oxygen in the blood and hind-gut water, which we have seen is adequate for the metabolic requirements for about seventy minutes, but also the oxygen dissolved in the water enclosed in the burrow. An average burrow is about one hundred centimeters long and two centimeters in diameter. It would contain some 314 cc. of water, and if this were saturated with air about 1.76 cc. of oxygen. This would last 135 minutes if used at a rate of 0.013 cc. per minute. The total oxygen supply of *Urechis* during low tide is sufficient for only about three hours according to these calculations.

In order to throw more certain light on the state of affairs during low tide, a series of analyses on the oxygen content of the water in the burrows was made. A rubber tube was thrust down into the burrows and sufficient water for analysis by the Winkler method (70 cc.) drawn out and transferred to a glass-stoppered bottle without exposure to air. A new burrow was selected for each observation. The flats had already become bare when we arrived but had not been so for more than one-half hour to judge from the state of the tide when the first observation was made. The last observations were made from the last burrows to be covered after the greater part of the flat was submerged by the rising tide. The oxygen content of the water left in a puddle by the receding tide, which serves to give an idea of the content of the burrow water before the flat was bared, was 0.34 volumes per cent. This relatively low value may be accounted for by the fact that the observations were made at daybreak. The water had been overnight in an estuary teeming with animal and vegetable life and oxygen losses had not been compensated by photosynthesis. The temperature of the water in the burrows was 15° C. at 6:08 A.M. and had risen to 17° C. at 11:30. At this time the water in the channel was 19° C. The results are recorded in Table XIV.

During the first hour after the flat is bare the oxygen content of the burrow water appears to decrease rapidly and at about the rate expected from the foregoing calculations. There is some irregularity in the

TABLE XIV

Oxygen Content of Water from Urechis Burrows During Low Tide

Time Sample Collected	Approximate Time Since Flat Became Bare	Oxygen Content of Water	
		From Individual Burrows	Mean
	hours	*cc. per 100 cc.*	*cc. per 100 cc.*
6:08 A.M.	0.5	0.21	0.21
6:24	0.7	0.16	0.16
6:30	0.9	0.13	0.13
6:35	1.0	0.15; 0.11; 0.11	0.12
7:40	2.0	0.24	0.24
7:55	2.2	0.16	0.16
8:00	2.5	0.12	0.12
9:30	4.0	0.06; 0.06; 0.06	0.06
10:30	5.0	0.16; 0.14; 0.14	0.14
11:15	5.7	0.06; 0.16; 0.23; 0.27; 0.27	0.20

individual measurements made during the third hour, but by the fourth hour the oxygen content has definitely sunk to a minimal value of 0.06 volumes per cent. During the fifth hour there is a perfectly definite increase in the oxygen content of the water in almost all of the burrows examined. These measurements support the view that the oxygen in the water inclosed within the burrow and in the blood is insufficient to maintain the normal metabolic rate for the duration of the low tide. After the first hour the oxygen in the burrow water diminishes rather slowly and one must conclude, either that the rate of oxygen consumption by the worm is diminished or that the oxygen in the burrows is replenished by some means. There is reason to believe that both these processes occur. It is well established that the metabolic rate of many marine organisms varies with the oxygen pressure in the environment (Amberson, Meyerson and Scott, 1924; Hall, 1929, and others). The measurements made on the water of the burrows very definitely indicate an increase in the oxygen content during the last hour before the flats were covered. This suggests that the water in the burrows is slowly replaced by the water with which the sand is permeated. The effect is probably related to changes of hydrostatic pressure within the flat occasioned by changes in the tide level, for it is reported that wells in sandy soil near the sea sometimes display definite changes in level related to the tides. The effect becomes noticeable only during the last hour when the tide is rising rapidly. It is presumably occurring throughout the low tide period and serves to check the exhaustion of the oxygen content of the water by the metabolism of the worms. If this view is correct it serves to explain how *Urechis* can withstand the 18 hours of low water which occur during the spring tides. At these times the small intermediate tide, although unable to cover the flats, will serve to move about the water within the flats and thus replenish to a certain degree the oxygen within the burrows of *Urechis*.

The oxygen content within the burrow never appeared less than 0.06 volumes per cent, which corresponds to an oxygen pressure of about fourteen millimeters. At this pressure the hemoglobin of *Urechis* is nearly 60 per cent saturated. During the greater part of the low tide the pressure of oxygen in the burrow is such that the hemoglobin of the blood will function effectively as an oxygen carrier while very little oxygen will be present in solution in the blood. Provided the oxygen in the burrows does not sink below the observed levels, the hemoglobin of the blood may be expected to transport an adequate supply of this gas to the organs of the body.

SUMMARY

1. The blood of *Urechis caupo* contains hemoglobin enclosed in corpuscles. The oxygen capacity of the blood varies from 2.66 to 7.22 volumes per cent and the percentage of cells in the blood from 18 to 40.

2. The oxygen dissociation curve is measured. Its position does not appear to be influenced by the carbon dioxide pressure. The effect of temperature upon the oxygen dissociation curve is of the direction and order observed in other bloods containing hemoglobins.

3. The carbon dioxide dissociation curve is measured. The ability of the blood to combine with carbon dioxide does not appear to be influenced by the degree of oxygenation of the blood.

4. The buffer value of the blood is 11 volumes per cent and is constant over a considerable range of carbon dioxide pressures. The concentration of hemoglobin accounts for the entire buffer effect.

5. Carbon dioxide is about equally distributed (in concentration) between the corpuscles and plasma. The plasma contains at most a negligible quantity of buffer material. With increased carbon dioxide tension there is a small, but distinctly limited exchange of material between the corpuscles and plasma which increase the ability of the latter to combine with carbonic acid.

6. The hemoglobin *in vivo* is almost completely saturated, but the pressure of oxygen in the blood may be considerably less than that in the surrounding water. The carbon dioxide content *in vivo* is 7 and 9 volumes per cent, corresponding to a carbon dioxide pressure of about seven millimeters Hg. The reaction of the blood is estimated to be about pH 7.1.

7. The "ventilation" of the respiratory organ, the hind-gut, is

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considered quantitatively, the result indicating that the respiratory activity is nicely adjusted to the metabolic requirements.

8. The function of hemoglobin and its relation to the oxygen supply during low tide are discussed. It is suggested that the movement of water within the flats due to changing tidal level is important in supplying oxygen when the tide is out.

REFERENCES

Amberson, W. R., H. S. Meyerson, and W. J. Scott, 1924. *Jour. Gen. Physiol.,* **7**: 171.

Barcroft, J., and H. Barcroft, 1924. *Proc. Roy. Soc.,* Ser. B., **96**: 28.

Bohr, C., 1897. *Ann. de Physik. u. Chemie,* 62: 644.

Brown, W. E. L., and A. Y. Hill, 1923. *Proc. Roy. Soc.,* Ser. B, **94**: 297.

Dill, D. B., and H. T. Edwards, 1931. *Jour. Biol. Chem.,* **90**: 515.

Drastich, L., 1928. *Compt. rend. Soc. de Biol.,* **99**: 991.

Fisher, W. K., and G. E. MacGinitie, 1928. *Ann. and Mag. Nat. Hist.,* Ser. 10, **1**: 199 and 204.

Fox, H. M., 1926. *Proc. Roy. Soc.,* Ser. B, **99**: 199.

Hall, F. G., 1929. *Am. Jour. Physiol.,* **88**: 212.

Hall, F. G., and I. E. Gray, 1929. *Jour. Biol. Chem.,* **81**: 589.

Hári, P., 1917. *Biochem. Zeitschr.,* **82**: 229.

Hastings, A. B., and J. Sendroy, Jr., 1925. *Jour. Biol. Chem.,* **65**: 445.

Henderson, L. J., 1928. Blood, A Study in General Physiology. New Haven.

Jordan, H., and B. Schwartz, 1920. *Pflüger's Arch.,* **185**: 311.

Kennedy, R. P., 1926–27. *Am. Jour. Physiol.,* **79**: 346.

Leitch, I., 1916. *Jour. Physiol.,* **50**: 370.

Macela, I., and A. Seliškar, 1925. *Jour. Physiol.,* **60**: 428.

Parsons, T. R., and W. Parsons, 1923. *Jour. Gen. Physiol.,* **6**: 153.

Seitz, 1907. *Zoöl. Jahrbuch.,* Abt. Anat., **24**: 323.

Van der Lingen, J., and L. Hogben, 1928. *Trans. Roy. Soc. S. Africa,* **16**: 205.

Van Slyke, D. D., A. B. Hastings, C. D. Murray, and J. Sendroy, Jr., 1925. *Jour. Biol. Chem.,* **65**: 701.

Vlès, F., 1923. *Arch. Phys. Biol.,* II, No. 6.

Winterstein, H., 1909. *Biochem. Zeitschr.,* **19**: 384.

OXYGEN AND CARBON DIOXIDE TRANSPORT BY THE BLOOD OF THE URODELE, AMPHIUMA TRIDACTYLA

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This paper is a presentation of the oxygen dissociation curves and of the carbon dioxide absorption curves of the blood of *Amphiuma tridactyla* together with comparisons with similar curves from the literature of the carp, the turtle, the frog, and man. In particular, the applicability of Hill's equation to the bloods of these species and the shape of the oxygen dissociation curve as an adaptive mechanism are briefly discussed. In addition, the properties of the carbon dioxide absorption curves, especially of those which are a result of the low hemoglobin content, are brought out.

TECHNIC

The blood was drawn from the heart 3–5 minutes after the injection of one cc. of 1:1000 heparin in 0.7 per cent sodium chloride to prevent clotting of the blood in the syringe during withdrawal. It was then transferred to a glass container coated with sufficient sodium fluoride and sodium oxalate to make a final concentration of about 0.1 to 0.2 per cent and kept at 2–8° C. until used.

The equilibration of the blood with the desired tensions of oxygen and of carbon dioxide, for 30 minutes at 22–26° C., was performed by a technic similar to that of Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (1922). Blood gases were determined in duplicate by the method of Van Slyke and Neill (1924). Analyses of the gas phase were made by the Haldane-Henderson apparatus. Hematocrite determinations of corpuscular volume were made before and sometimes after samples were drawn for equilibration. No difficulty was encountered in the use of the ferricyanide method for *Amphiuma* blood in contrast with the experience of Krogh and Leitch (1919) with fish blood. Caprylic alcohol as an antifoamer was omitted because of clot formation.

OXYGEN CONSUMPTION OF AMPHIUMA BLOOD

The spontaneous diminution of the oxygen of blood so common particularly with nucleated cells is quite marked in *Amphiuma* blood. It was found (Table I) that keeping the blood at 2–5° C. except during the period of actual equilibration practically eliminated this spontaneous oxygen consumption. The presence of physiological amounts of carbon dioxide apparently diminishes appreciably the oxygen loss. The slight loss which may occur during 20–30 minutes of equilibration and handling is negligible. Wastl (1928) has successfully used KCN to prevent oxygen consumption in the blood of the carp, but its effect in *Amphiuma* blood appeared variable and we abandoned its use in favor of cold to eliminate this source of error.

Table I

Effect of Temperature on the Oxygen Consumption of Amphiuma Blood

Temperature = 20° C.

Time *minutes*	Oxygen Content *vol. per cent*
0	2.64
59	1.41
118	0.20
197	0.0

Temperature = 3° C.

Time *minutes*	Oxygen Content at Varying CO_2 Tensions					
	1–3 mm.	25 mm.	42 mm.	44 mm.	53 mm.	60 mm.
0	3.49	4.64	3.00	4.57	4.52	6.23
20			2.98		4.52	6.18
60	3.16	4.70	2.64	4.41	4.46	5.94
120	2.89	4.61	2.55	4.23	4.19	
180	2.69	4.50	2.69	3.99		
240	2.69	4.59				

The Solubility Coefficient of Oxygen in Amphiuma Blood

It is usually assumed as a sufficient approximation that the solubility of a gas in blood or serum, relative to its solubility in water is proportional to the water content of the blood. For *Amphiuma* blood the relative solubilities in serum and in whole blood accordingly would be about 95 per cent and 87 per cent respectively. We have measured αO_2 directly in both serum and whole blood of *Amphiuma* in the following way: Samples of serum and of whole blood were equilibrated with air and with 99.6 per cent oxygen. To diminish spontaneous oxygen consumption the equilibration was performed at 4° C. and the solubility cœfficient relative to that of water at the same temperature calculated. After equilibration blood was drawn into a mercury receiver. Sampling and transfer to the Van Slyke apparatus was accomplished by using a Barcroft pipette. Assuming that the oxygen of the air is sufficient to fully saturate the hemoglobin, αO_2 may be calculated from the difference in the oxygen content of the samples by the equation:

$$\alpha O_2 = \frac{760(V - V')}{P - P'},$$

where V and V' are respective oxygen contents in volume percentage and P and P' are respective oxygen partial pressures in mm. Hg. Table II shows that the relative solubilities of oxygen in *Amphiuma* serum and whole blood are about 92 per cent of that in water; and this figure is used in the subsequent calculations. These figures fail to demonstrate a difference in the αO_2 between serum and whole blood of *Amphiuma,* which is not surprising in view of the low corpuscular volume of the blood.

TABLE II

Solubility Coefficient of Oxygen in Amphiuma Whole Blood and Serum

Temperature		Oxygen Tension	Oxygen	αO_2
° C. ± .5°		*mm. Hg*	*vol. per cent*	
Serum	3	749	3.90	0.0393
		157	0.84	
	6	754	3.98	
		158	0.79	0.0408
	3	754	3.98	0.0401
	3	748	4.04	
		157	0.83	0.0403
	4	157	0.84	0.0410
Av.	4	751	3.97	
		157	0.82	0.0403
		Average αO_2 = 92% of αO_2 water.		
Whole blood	4	765	12.23	
		161	9.05	0.0400
	4	764	12.28	
		161	9.05	0.0406
	4	740	12.90	
		156	9.77	0.0398
Av.	4	756	12.45	
		159	9.29	0.0402
		Average αO_2 = 92% of αO_2 water.		

HEMOGLOBIN CONTENT OF THE CELLS

The corpuscular volume varies considerably and is slightly higher than that found by Southworth and Redfield (1926) for the turtle. The oxygen capacity also varies greatly in *Aphiuma* blood. In Table III it is seen that the ratio of oxygen capacity to cell volume is roughly constant, and averages 0.25. The data of Southworth and Redfield (1926) on the turtle show an approximate ratio of oxygen capacity to cell volume of 0.50 and the average figure for human blood is said to be 0.45. The red cells of both *Amphiuma* and the turtle are nucleated and as a rough approximation of the fraction of the volume occupied by the cytoplasm we take 0.80, and calculate the concentration of hemoglobin per unit volume of cytoplasm. It appears from these data that the concentrations of hemoglobin in the cytoplasm of the red cells of *Amphiuma* and the turtle are 0.32 and 0.63 volumes of oxygen per volume of cytoplasm respectively, or 71 per cent and 140 per cent of the concentration of hemoglobin in the cytoplasm of the human red cell, an interesting divergence in this factor for the three different species.

TABLE III

Variation in Oxygen Capacity and in Corpuscular Volume in the Blood of Amphiuma and the Ratio of Oxygen Capacity to Cell Volume

Red Cells *vol. per cent*	Oxygen Capacity *vol. per cent*	Oxygen Capacity / *cell volume*
14	2.52	0.18
15	4.52	0.30
17	4.7	0.28
20	5.01	0.25
20	5.8	0.29
25	6.1	0.24
28	8.38	0.30
36	9.64*	0.27
52	9.42*	0.18

* Concentrated blood.

THE OXYGEN DISSOCIATION CURVE

The oxygen-binding properties of both hemoglobin solutions and bloods, except at low and high degrees of saturation, may be represented with sufficient accuracy by the equation of Hill (1910):

$$\frac{\mathrm{HbO_2}}{\mathrm{Hb}} = K_n P^n.$$

We have found it convenient to analyze our data on *Amphiuma* blood by means of this equation and it is evident that it holds quite well for this blood as shown by Fig. 1. Here are plotted the points of an oxygen dissociation curve at 43 ± 3 mm. of carbon dioxide (Table IV) and for comparison, points from similar data for the carp (Wastl, 1928), turtle (Southworth and Redfield, 1926), and man (Bock, Field and Adair, 1924). The respective curves are calculated from the values of

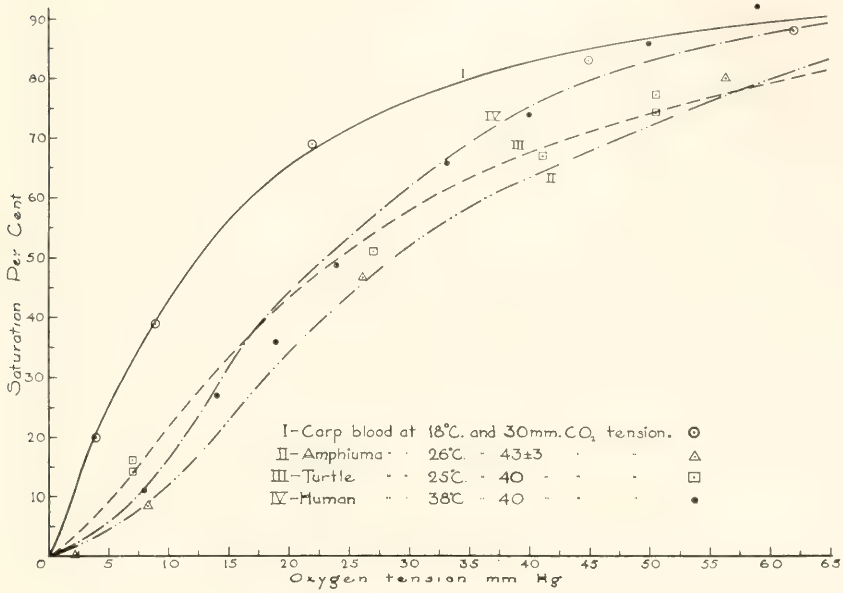

FIG. 1. Oxygen dissociation curves. The lines are calculated by Hill's equation and plotted using the n and K_0 values (Table V) from the original data (points).

n and K_0 for the blood of each species, given in Table V. These constants in turn were obtained in the usual way by plotting log HbO_2/Hb against log PO_2, as in all cases, the points between approximately 10 to 90 per cent saturation fall quite closely on a straight line represented by the equation,

$$\log \frac{HbO_2}{Hb} = \log K_0 + n \log P,$$

from which the constants K_0 and n have been calculated. The conformity of the experimental observations (points) with the equation of Hill (lines) shows that Hill's equation holds reasonably well within the limits specified for these divergent species. *Amphiuma* resembles human blood in showing an " S " shaped oxygen dissociation curve which is absent in the carp and turtle. It is well known that the " S " shaped curve is associated with high values of N (greater than 1.8). This is apparent from the comparison of the curves of these four

TABLE IV

Oxygen Dissociation Curve of *Amphiuma* Blood. Temperature = 26° C. Carbon dioxide tension = 43 ± 3 mm. Oxygen capacity = 8.4 vol. per cent. $\alpha O_2 = 0.92\ \alpha O_2$ (in water).

	O_2 Tension	HbO_2	Saturation
	mm. Hg	*vol. per cent*	*per cent*
1.	2.2	0.01	0.0
2.	8.4	0.70	8.3
3.	15.8	2.2	26.3
4.	26.2	3.9	47.0
5.	56.4	6.7	80.0
6.	79.6	7.6	91.0
7.	143.5	8.4	100.0

forms whose n values vary from 1.4 to 2.0. The absence of the " S " shape has been interpreted by Krogh to signify an adaptation of bottom forms such as the carp to low oxygen tension, whereas the free-swimming forms, *e.g.*, the trout, whose curves are " S " shaped, do not possess this adaptation. The air-breathing *Amphiuma,* despite its habit of remaining submerged for considerable periods, appears not to possess this adaptation. That the " S " shaped curve is not a necessary property of the blood of air-breathing animals is evident from its absence in the turtle, whose n value is 1.5.

TABLE V

Hill's n and K_0 for Carp, Turtle, Man, and Amphiuma Whole Blood

Species	n	$K_0 \times 10^{-3}$	
Carp	1.3	36.0	Wastl (1928)
Amphiuma	1.8	2.5	
Turtle	1.5	8.3	Southworth and Redfield (1926)
Man	2.0	1.9	Bock, Field, and Adair (1924)

EFFECT OF CARBON DIOXIDE ON THE OXYGEN DISSOCIATION CURVE

The increased acidity of the blood with increased carbon dioxide tension has been abundantly shown to decrease the oxygen saturation at a given tension. In other words, K_0 is decreased by increasing carbon dioxide tension. That this effect is manifest in *Amphiuma* is shown by the data of Table VI. The values of K_0 have been calculated by Hill's equation, using $n = 1.8$. This decrease of the affinity of hemoglobin for oxygen with increased acidity is in accord with experience in many species such as man, dog, fishes, and turtle.

TABLE VI

Effect of Carbon Dioxide on K_0 of Amphiuma Blood

	Oxygen Capacity	CO_2 Tension	Oxygen Tension	Saturation	$K_0 10^{-3}$
	vol. per cent	*mm. Hg*	*mm. Hg*	*per cent*	
1.	5.8	*a.* 2.3	53.0	88.0	5.7
		b. 38.0	83.0	84.0	1.8
2.	4.5	*a.* 1.2	26.0	58.0	3.9
		b. 43.5	85.0	87.0	2.2
3.	2.5	*a.* 1.9	44.0	78.0	4.2
		b. 42.7	48.0	62.0	1.5

THE TRANSPORT OF CARBON DIOXIDE

Figure 2, made from the data of Table VII, shows three carbon dioxide absorption curves of oxygenated *Aphiuma* blood. Curve I is characterized by a rather low CO_2 capacity, about 39 volumes per cent at 70 mm. CO_2 tension, and also by the fact that when this blood is equilibrated with gas mixtures lacking carbon dioxide, very little of this gas remains in the blood. Curves II and III, on the other hand, are

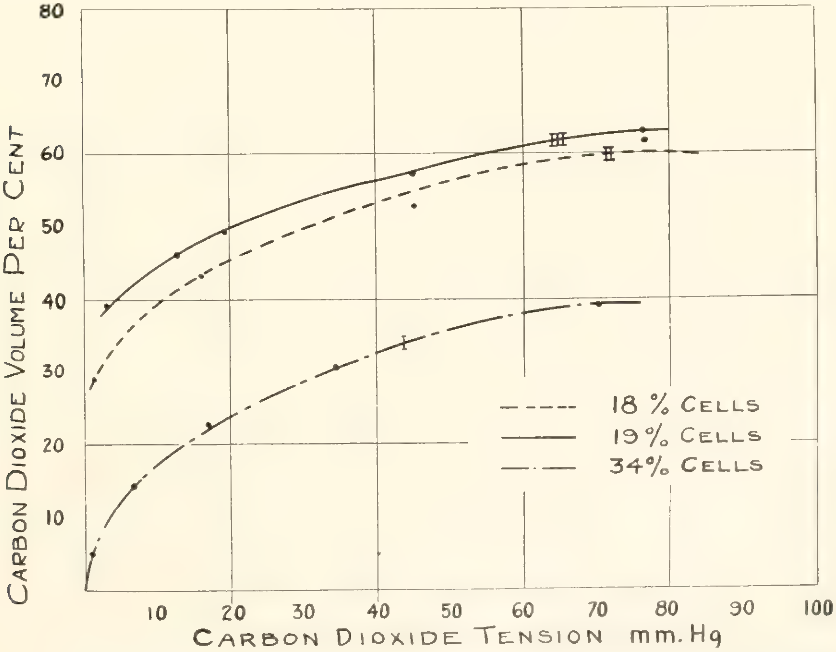

FIG. 2. Carbon dioxide absorption curves of oxygenated *Amphiuma* blood. Temperature 24 ± 2° C.

characterized by a much higher carbon dioxide capacity, approximately 63 volumes per cent at 78 mm. carbon dioxide tension, and further by the fact that when these bloods are exposed to low carbon dioxide tension during equilibration a relatively large amount of carbon dioxide, 30–40 volumes per cent, still remains in the blood. The considerable difference between curve I and curves II and III is probably due to the large variation in hemoglobin content. It is well known that hemoglobin functioning as an acid can combine with only a limited amount of base so that only limited amounts of $NaHCO_3$ will be decomposed into sodium hemoglobinate and carbon dioxide when blood which contains but a limited amount of hemoglobin is exposed to zero or low tension of carbon dioxide.

TABLE VII

Carbon Dioxide Absorption Curves. (See Fig. 4.) Temperature = 24 ± 2° C. Oxygenated blood.

Curve No.	Point No.	CO_2	CO_2
		mm. Hg	*vol. per cent*
I (Cells = 34 per cent)	1.	1.1	4.48
	2.	6.7	14.08
	3.	16.9	22.36
	4.	34.4	30.25
	5.	70.3	38.85
II (Cells = 19 per cent)	1.	3.4	39.45
	2.	12.9	46.11
	3.	19.5	48.79
	4.	45.4	56.78
	5.	76.1	62.94
III (Cells = 18 per cent)	1.	1.1	28.62
	2.	16.3	43.0
	3.	45.5	53.32
	4.	76.4	61.05

The amount of sodium bicarbonate which will be decomposed by the hemoglobin in passing from a definite tension, *e.g.* 40 mm. of carbon dioxide, to a tension of zero may be calculated as follows: let the maximum base bound per unit of hemoglobin be b_0 and the base bound at 40 mm. carbon dioxide per unit of hemoglobin be b. Then if [Hb] be the concentration of hemoglobin in the blood, the $NaHCO_3$ decomposed in the reaction—

$$NaHCO_3 + HHb \rightarrow NaHb + H_2CO_3$$

is obviously $\triangle\ NaHCO_3 = (b_0 - b) \cdot [Hb.]$ A rough extrapolation of the curves of Hastings, Sendroy and Heidelberger (1924) for horse hemoglobin gives $b_0 = 8$ and $b = 2$. That is, blood in passing from 40 mm. to zero millimeters of carbon dioxide tension will decompose no more than $b_0 - b$ volumes of $NaHCO_3$ for each volume per cent of oxygen capacity. For the *Amphiuma* bloods whose carbon dioxide absorption curves are shown in Fig. 2 the oxygen capacities are respectively 8.5, 4.5 and 4.5 volumes per cent. The corresponding amounts of $NaHCO_3$ decomposable are therefore 51, 27 and 27 volumes per cent. In the case of curve I, however, the amount of $NaHCO_3$ at 40 mm. was 32 volumes per cent which, being less than the maximum of 51, will be entirely decomposed at zero carbon dioxide tension. This was found to be the case as shown in Fig. 2. In the case of curves II and III, where at 40 mm. carbon dioxide tension the $NaHCO_3$ is approximately 55 volumes per cent, only 27 volumes per cent will be decomposed. This was found to be approximately the case as seen in Fig. 2. This calculation, of course, is admittedly the roughest sort of approximation from insufficient data and is offered only as a semi-quantitative explanation of the phenomenon of incomplete decomposition

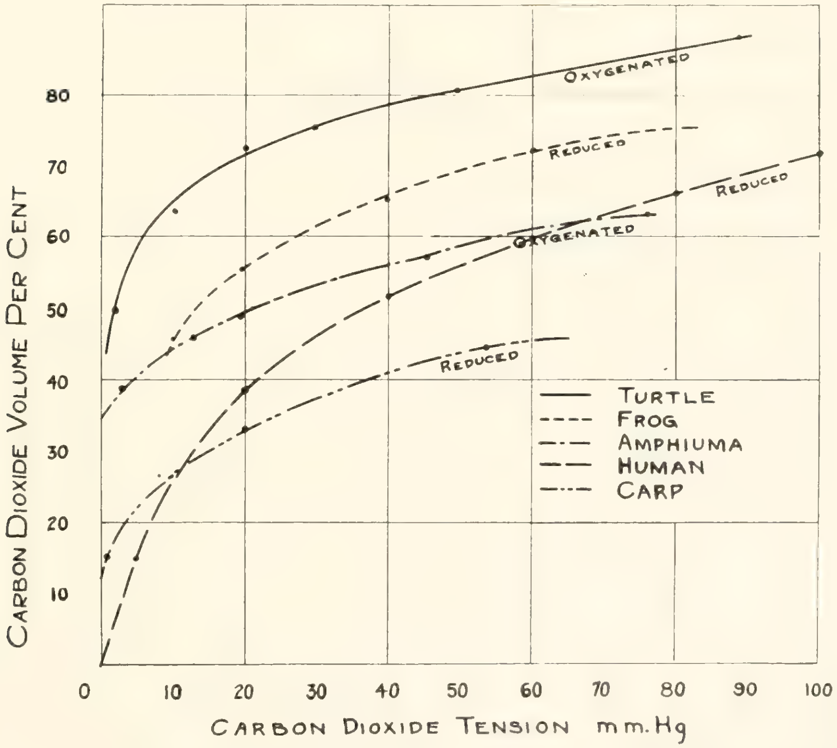

FIG. 3. Carbon dioxide absorption curves of carp, frog, *Amphiuma*, turtle and man. Temperature, human curve 38° C., all others, room temperature.

of $NaHCO_3$ by hemoglobin at zero millimeters of carbon dioxide tension. A quite similar qualitative explanation of the same phenomenon in turtle blood was first offered by Southworth and Redfield (1926).

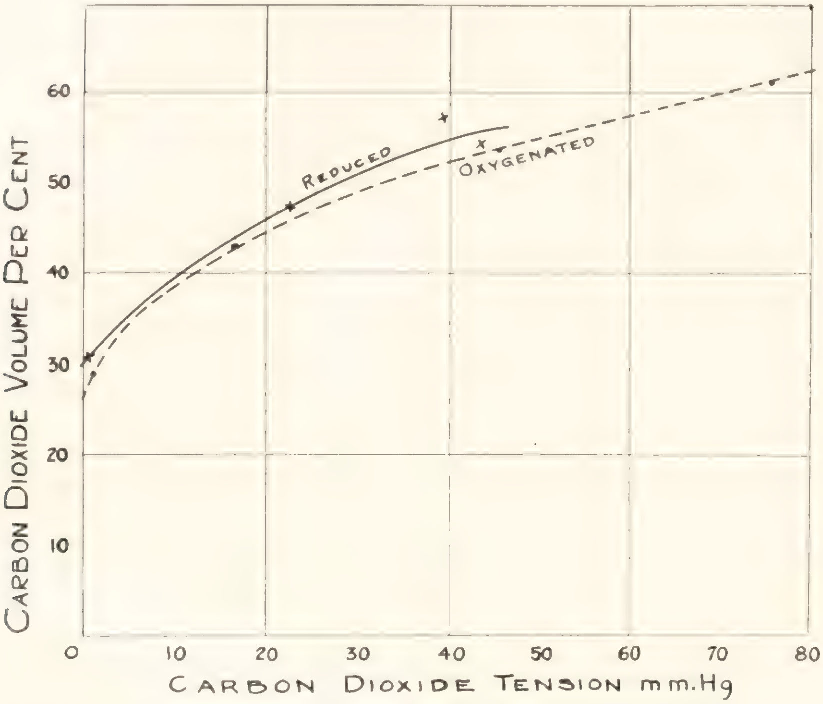

Fig. 4. The effect of oxygenation and reduction on the carbon dioxide absorption curve of *Amphiuma* blood. Eighteen per cent cells. Temperature 24° C.

Figure 3 shows a number of carbon dioxide absorption curves for the carp, frog, *Amphiuma*, turtle, and man, representative of four of the classes of vertebrates. All of the curves for the lower vertebrates resemble each other more than they do the mammalian curve. In these few examples the amphibia occupy an intermediate position with respect to carbon dioxide content between the teleostian carp and the reptilian turtle. The marked flatness of the turtle curve is attributed by Southworth and Redfield (1926) to the low corpuscular volume, *i.e.*, the low Hb of turtle blood. Wastl (1928) gives the same sort of explanation for the essentially parallel curve for carp blood. A similar explanation for the flatness of the *Amphiuma* curve is indicated just as it is for the other lower vertebrates, since the blood of all of these animals shows a corpuscular volume only about one-half to one-third that of human blood.

THE EFFECT OF OXYGENATION AND REDUCTION OF THE BLOOD ON THE CARBON DIOXIDE TRANSPORT

Figure 4 shows the difference in the amounts of carbon dioxide carried by oxygenated and reduced *Amphiuma* blood. The difference in carbon dioxide content in the two cases is not so great for *Amphiuma* blood as that found by Christiansen, Douglas, and Haldane (1914) for human blood. In fact, this difference amounts to about two volumes per cent at physiological levels for *Amphiuma,* while for man the corresponding value is about 5.5 volumes per cent. If the difference in the amounts of carbon dioxide carried by oxygenated and by reduced blood be divided by the oxygen capacity of the sample of blood, a ratio $\frac{\Delta CO_2}{\Delta O_2}$, *i.e.,* the increase of carbon dioxide content per unit of oxygen capacity, is obtained. The value of this ratio in the case of human blood is about 0.28 volume of carbon dioxide per unit volume of oxygen capacity. In five experiments we attempted to determine this ratio closely, but our results were quite divergent. We found for *Amphiuma* blood values of $\frac{\Delta CO_2}{\Delta O_2}$ as follows: 0.23, 0.31, 0.46, 0.71, and 0.93, averaging 0.54. It is obvious that the calculation of this ratio is subject to considerable error since it is the ratio of small differences of large volume; nevertheless, the results are all in the same direction as in the blood of man. The mean value of the ratio for *Amphiuma* is, of course, very approximate. Physiologically, however, oxygenation and reduction have little effect on the transport of carbon dioxide by *Amphiuma* blood. In this respect, also, the *Amphiuma* is like the turtle. The data for the curves of Fig. 4 are included in Table VIII.

TABLE VIII

The Effect on Carbon Dioxide Content of Oxygenation and Reduction of Amphiuma Blood. (See Fig. 4.) Temperature = 24° C.

Curve No.	Point No.	CO_2 Tension	CO_2
		mm. Hg	*vol. per cent*
I (Reduced blood, O_2 capacity 4.4 vol. per cent)	1.	0.7	30.87
	2.	22.7	47.28
	3.	39.8	56.94
	4.	43.6	54.34
II (Oxygenated blood, O_2 capacity 4.4 vol. per cent)	1.	1.1	28.62
	2.	16.3	43.0
	3.	45.5	53.32
	4.	76.4	61.05

Summary

1. The corpuscular volume of *Amphiuma* blood varies considerably, from 12 to 35 per cent.

2. The oxygen capacity varies from 3 to 10 volumes per cent.

3. It is shown that the oxygen dissociation curve is like the typical mammalian curve with certain features in common with those of the turtle and carp.

4. The presence of physiological amounts of carbon dioxide affects the oxygen dissociation curve in the usual way.

5. The comparative values of n and K_0 of the Hill equation are given for the blood of *Amphiuma,* carp, turtle and man, and the equation of Hill for these bloods is shown to hold within the limits specified.

6. The mechanism for the transport of carbon dioxide in the *Amphiuma* blood is much like that in the turtle and the flatness of the carbon dioxide absorption curve is explained as a function of the limited amount of hemoglobin.

7. The difference in carbon dioxide carried by oxygenated and reduced blood is quite small and probably has little physiological significance, though the increase in carbon dioxide content per unit of oxygen capacity is in the same direction as that for man.

The major portion of this work has been carried out under the direction of Dr. William C. Stadie. I wish to thank him for his interest in this problem and for his continual aid and encouragement.

BIBLIOGRAPHY

Austin, J. H., G. E. Cullen, A. B. Hastings, F. C. McLean, J. P. Peters, and D. D. Van Slyke, 1922. *Jour. Biol. Chem.,* **54**: 121.

Bock, A. V., H. Field, Jr., and G. S. Adair, 1924. *Jour. Biol. Chem.,* **59**: 353.

Bohr, Christian, 1905. *Skand. Arch. f. Physiol.,* **17**: 104.

Bohr, C., K. Hasselbalch, and A. Krogh, 1904. *Skand. Arch. f. Physiol.,* **16**: 402.

Christiansen, J., C. G. Douglas, and J. S. Haldane, 1914. *Jour. Physiol.,* **48**: 244.

Hastings, A. B., Julius Sendroy, C. D. Murray, and Michael Heidelberger, 1924. *Jour. Biol. Chem.,* **61**: 317.

Hill, A. V., 1910. *Jour. Physiol.,* **40**: iv–vii, Proceedings of 1910.

Krogh, A., and I. Leitch, 1919. *Jour. Physiol.,* **52**: 288.

Southworth, F. C., Jr., and A. C. Redfield, 1926. *Jour. Gen. Physiol.,* **9**: 387.

Van Slyke, D. D., and J. M. Neill, 1924. *Jour. Biol. Chem.,* **61**: 523.

Wastl, H., 1928. *Biochem. Zeitschr.,* **197**: 363.

Wastl, H., and A. Seliškar, 1925. *Jour. Physiol.,* **60**: 264.

MOVEMENT AND RESPONSE IN DIFFLUGIA WITH SPECIAL REFERENCE TO THE NATURE OF CYTOPLASMIC CONTRACTION[1]

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INTRODUCTION

It has been repeatedly observed by various investigators that when *Difflugia* and other shelled rhizopods travel, pseudopods, one after another, extend in a given direction, become attached at the tip to the substratum, then shorten and pull the shell forward; but the only reference concerning the mechanism involved in these processes is found in a former paper in which I came to the following conclusions (1926, p. 413): "In this process of locomotion the tip of the attached pseudopod functionally becomes the posterior end. The plasmagel probably changes into plasmasol here and then flows directly into the new pseudopod. . . . The extension of the pseudopods is . . . dependent upon contraction in the plasmagel, resulting in local pressure on the plasmasol." However, in this work but little evidence in support of these conclusions was obtained from observation on *Difflugia*. They were largely based upon the results of detailed observations on the process of locomotion in *Amœba*. Fortunately, I have recently had the opportunity, under very favorable conditions, to make equally detailed observations on the process of locomotion in *Difflugia*. These observations are considered in the following pages.

MATERIAL AND METHODS

Two species of *Difflugia* were used in this investigation: *D. pyriformis* (Leidy) and *D. acuminata* (Leidy). Both were found in the ooze on the bottom of a large permanent pond, the edges of which were frequented by cattle and horses. *Pyriformis* was abundant, *acuminata* rather scarce. The pond is located near Town Hill, Mt. Desert Island, Maine.

All of the specimens studied were well filled with *Chlorella*.

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[1] Contribution from the Mt. Desert Island Biological Laboratory. I am greatly indebted to the Director of this laboratory for excellent laboratory facilities and to the Research Corporation for financial aid in procuring assistance in the investigation.

They were very active and they lived well in the laboratory, both in jars and on slides under cover-glasses sealed with vaseline. They were consequently very favorable for making extensive observations on locomotion.

The process of locomotion was studied under Zeiss apochromatic objectives and compensating oculars with magnifications ranging from 200 to 1200 diameters. With the lower magnifications the specimens were observed in watch-glasses, with the higher under cover-glasses supported and sealed with vaseline. In some observations the cover-glass was far enough from the slide so that the specimens could move about freely; in others it pressed on the shells of the difflugiae just enough to prevent locomotion, and in still others it pressed on the shells so much that they broke, some slightly, others considerably. After the shells were broken some of the specimens left and moved about naked. The process of locomotion was thus studied in specimens with shells and in specimens without shells.

The response to tactile and photic stimulation was also briefly studied as indicated below.

Locomotion

Difflugia pyriformis with Shells Free

The shell of *Difflugia pyriformis* is flask-shaped but usually considerably flattened. It consists of a layer of sand grains which vary greatly in size. The interstices between the grains of sand are filled with a yellowish substance which holds them together. According to Leidy, the shells vary greatly in size, ranging from .06 to .58 mm. in length and from .04 to .24 mm. in width. In the specimens studied the shell was about .4 mm. long and .2 mm. wide.

When at rest the living portion of *Difflugia* is usually entirely within the shell and it fills only about three-fourths of the space. When it begins to move a pseudopod forms on the surface of the body below the opening in the shell, then extends out through the opening and advances free into the surrounding medium, until it is about as long as the shell (Fig. 1). As the pseudopod advances it usually swings from side to side freely and extensively but not rapidly. The tip thus frequently moves through an arc of nearly 90° in a little more than one second. In this swinging the tip sooner or later comes in contact with the substratum to which it adheres. Then it contracts slowly and pulls the shell along. Before this pseudopod has disappeared another one usually develops at the opening of the shell and extends at a considerable angle with the old one, but as the old one disappears, the new one becomes directed forward, attaches and then contracts and pulls the shell forward. This process is repeated, one pseudopod developing after another and each pulling the shell forward .2 to .4 mm. Movement of the shell is consequently intermittent.

Sometimes the new pseudopod attaches before the old one detaches and occasionally two pseudopods extend simultaneously, become attached and contract simultaneously (Fig. 2*C*). Pseudopods also sometimes branch at the tip or near it and elsewhere. When a pseudopod is extending or advancing there is rapid streaming forward in the middle. This becomes slower and slower toward the surface and disappears entirely before the surface is reached, *i.e.*, there is a layer immediately below the surface which does not move forward. This layer is usually very thin and it is evidently relatively solid, *i.e.*, gel. It forms a tube through which the more fluid part, the plasmasol, flows. At the tip of the pseudopod, the plasmasol stream spreads out and as it comes in contact with the end of the plasmagel tube it gelates, *i.e.*, it is here transformed into plasmagel. The plasmagel tube is consequently built forward by gelation of plasmasol at the tip of the pseudopod just as it is in *Amœba*. But the plasmagel tube is usually open at the tip of the pseudopod and the plasmasol flows forward to the end with nothing corresponding to the hyaline cap in *Amœba* (Fig. 1).

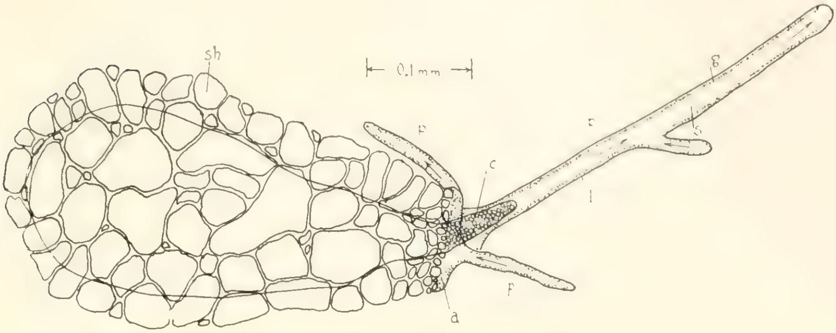

FIG. 1. Camera drawing of *Difflugia pyriformis* in locomotion. *sh*, shell constructed of sand grains cemented together; *p*, pseudopods; *l*, plasmalemma; *g*, plasmagel; *s*, plasmasol; arrows, direction of flow in the plasmasol; *c*, *Chlorella;* *a*, disc of cytoplasm projecting to the edge of the shell; *mm.*, scale showing magnification. The stippling of the plasmagel in this and the following figures is not intended to represent structure. The plasmagel contains numerous small granules, but the plasmasol contains an equal number of the same description.

The large region almost entirely within the shell, enclosed by the solid line, contained so many chlorellae that it was dense green. This region was covered with a thin layer of hyaline substance. The pseudopods usually contain but few chlorellae. At the mouth of the shell the hyaline layer (*h*) usually extends out over the edge of the shell forming a sort of cushion.

Note that the plasmasol extends to the tip of the pseudopod and that there is no hyaline cap.

The plasmagel and the plasmasol in the pseudopod contain innumerable small granules and after the pseudopod is fully extended, a group of chlorellae usually appear in the plasmasol at the base. These are, however, never carried to the tip where the plasmasol is transformed into plasmagel, and they consequently never get into and become a part of the plasmagel (Fig. 1).

The surface of the pseudopod is covered with a very thin membrane which is in fairly close contact with the plasmagel. This membrane cannot be seen directly, but the fact that the plasmasol flows to the very tip of the pseudopod and stops there, and the fact that hyaline blisters form in various regions on the surface, as will be demonstrated presently, show that there is a surface membrane, a plasmalemma. This doubtless slides over the plasmagel and stretches as the pseudopod extends. The pseudopod in *Difflugia* is therefore point for point perceptually the same in structure and in the process of extension as it is in *Amœba.*

Nothing could be directly ascertained concerning the mechanism involved in the extension of pseudopods in *Difflugia,* but the fact that they are not in contact with the substratum when they advance shows that they are pushed out by contraction of the portion of the body in the shell. This contraction is doubtless in the plasmagel, just as it is in *Amœba.*

The bending of the pseudopod from side to side is doubtless due to local contraction of the plasmagel on the side of the pseudopod toward which it bends. This contention is strongly supported by the results obtained in observations on response to contact, presented in a succeeding section of this paper.

As soon as the tip of the pseudopod comes in contact with the substratum it adheres to it. Then it flattens and spreads over the substratum and as it spreads it attaches. This continues until the attached surface has increased three or four times in width. Thus the tip of the pseudopod becomes very firmly fastened to the substratum (Fig. 2).

Immediately after the pseudopod has become attached, one or more blisters form at the point of adhesion to the substratum. These consist of droplets of fluid which apparently have been squeezed out of the plasmagel. The fluid aggregates between the outer surface of the plasmagel and the substratum and then spreads laterally (Fig 2*B*). This fluid is definitely differentiated from the surrounding medium, showing that there must be on the pseudopod a surface membrane, a plasmalemma, which has been separated from the plasmagel by the fluid squeezed out. Similar blisters are formed under other conditions as indicated below.

When such blisters form, fluid can actually be seen to flow from the plasmagel, which now becomes clearly visible as a thin granular layer. Under certain conditions this granular layer can be seen to break after the blister containing hyaline fluid has been formed (Fig. 2*A, B*); then the granular plasmasol flows through and disperses throughout the hyaline fluid. In this fluid there are a few scattered granules which are in violent Brownian movement. These facts show that the hyaline substance in the blister has liquid properties and that the plasmagel layer is relatively solid. After the blisters have fully formed and have spread over the substratum the Brownian movement in them ceases, indicating that the fluid in them has gelated. This conclusion is supported by other evidence presented below.

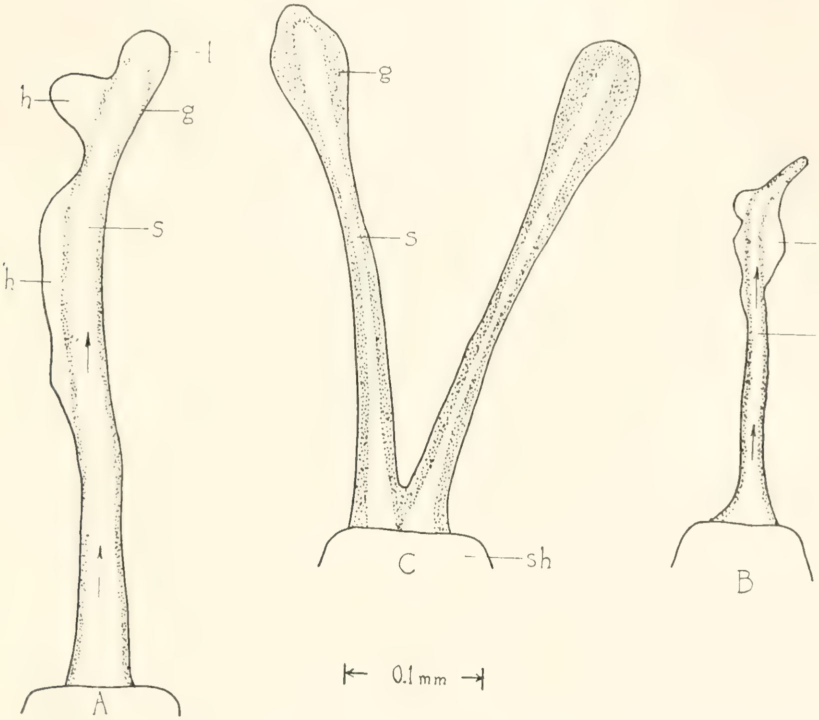

FIG. 2. Camera outlines of pseudopods of *Difflugia pyriformis*, illustrating the process of attachment. *A* and *B*, first stages in attachment; *C*, attachment complete; *h*, hyaline blisters; *s*, plasmasol; *g*, plasmagel; *l*, plasmalemma.

Note that during the process of attachment the hyaline layer in the region that becomes attached increases greatly in thickness and spreads out over the substratum and then gelates.

In the specimen represented in *C* two pseudopods extended and attached simultaneously. This does not often occur. In *C* the pseudopod attached some distance back of the tip.

Attachment of the pseudopod must be due to adhesive substance on the surface of the plasmalemma, or to adhesive character of the plasmalemma itself. The fluid in the blister formed immediately after the pseudopod becomes attached must be squeezed either out of the plasmagel in the region of adhesion, owing to local contraction, a process resembling syneresis; or out of the plasmasol, owing to local increase in the water permeability of the plasmagel and contraction in this layer elsewhere. The spreading out on the substratum and the flattening of the tip must be due to surface tension which pulls the edges of the pseudopod in all directions over the substratum in the same way that the edges of a drop of oil on water are pulled by surface tension over the surface of the water. If this obtains, the surface tension of the pseudopod-water interface plus that of the pseudopod-substrat interface must be less than the surface tension of the substrat water interface.

After the tip of the pseudopod is attached, the plasmagel becomes thicker and every portion of the pseudopod shortens, resulting in marked and fairly rapid decrease in the length of the pseudopod and in considerable increase in thickness. During the process of shortening of the pseudopod the plasmasol usually does not flow toward the shell as rapidly as the plasmagel retracts, *i.e.,* the plasmasol actually flows forward in relation to the plasmagel, although it is moving toward the shell. Sometimes, however, a branch forms near the tip of a pseudopod while it is contracting and when this occurs the plasmasol usually actually flows forward, *i.e.,* it flows forward in reference to the shell and in reference to points outside the organism. This shows that retraction of a pseudopod in *Difflugia* differs considerably from retraction of a pseudopod in *Amœba.* In the latter, as I have demonstrated elsewhere (1926), the pseudopods shorten, owing to transformation of plasmagel into plasmasol at the tip. In the former they shorten owing to contraction of the plasmagel throughout the entire length. This is, however, eventually followed by transformation of the plasmagel into plasmasol at the tip. Retraction of the contracted pseudopods in *Difflugia* is therefore like the retraction of the pseudopod in *Amœba.*

Sometimes pseudopods are fully extended and then retracted without having become attached. When this occurs retraction is usually much more rapid than it is when they are attached and after they have shortened considerably numerous small blisters appear scattered over the surface, except near the tip; but they become more and more abundant as one approaches the base of the pseudopods. Verworn (1889) observed the formation of similar blisters during retraction of pseudopods in *Difflugia urceolata.* These blisters increase in number as the pseudopods decrease in length (Fig. 3). They are, as will be demonstrated presently, associated with thickening of the plasmagel.

After a pseudopod has become attached, the fluid in the blisters in contact with the substratum gelates and the plasmagel throughout the entire length of the pseudopod thickens. This is, however, much more evident in specimens in which the shell is firmly held by the pressure of the cover-glass than it is in those in which the shell is free. I shall consider this matter more fully in the following section.

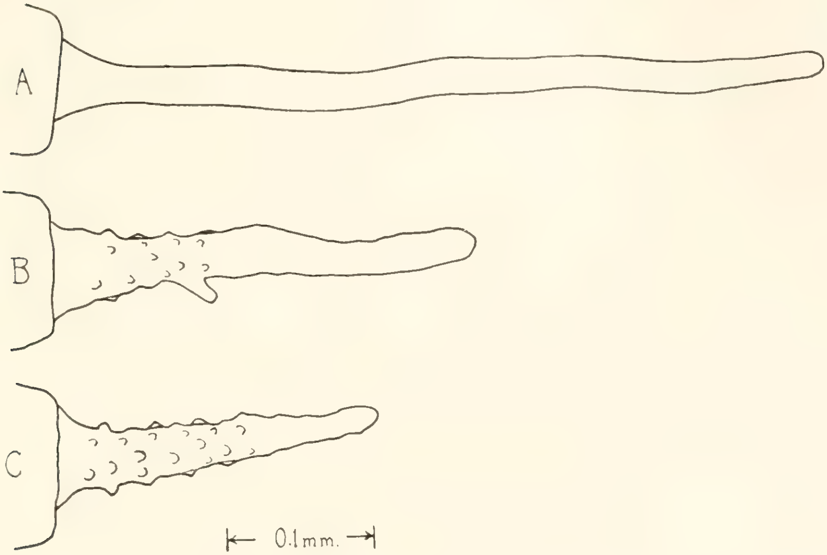

FIG. 3. Camera outlines of pseudopods of *Difflugia pyriformis,* illustrating contraction without attachment to the substratum. *A,* pseudopod fully extended; *B* and *C,* stages in contraction.

Note that during contraction numerous hyaline blisters form on the surface. This is due to localized thickening of the hyaline layer. The thickness of the plasmagel increases markedly when the hyaline blisters form.

Difflugia pyriformis with Shells Fastened to the Substratum

The shell of *Difflugia* was fastened and held as follows: Several specimens which were nearly the same in size were mounted in water under a cover-glass supported with a ridge of vaseline. Then water was slowly removed until the cover-glass pressed upon the shells just enough to prevent locomotion but not enough to break the shells. In specimens in this condition all the processes of locomotion observed in specimens with the shells free occur except movement of the shell, but there are some illuminating modifications in some of the processes.

Extension, bending and attachment of pseudopods are in all respects precisely the same under the two conditions, but the result of contraction is very different, the shell moving forward under the one condition and the attachment of the pseudopod breaking under the other.

After the pseudopod has become attached in specimens with the cover-glass resting on the shell, the plasmasol gradually becomes narrower and streaming in it, slower; the plasmagel becomes thicker, and the entire pseudopod some little distance back of the point of attachment becomes thinner. The portion of the pseudopod between the point of attachment and the shell becomes perfectly straight and numerous lines running parallel with the longitudinal axis of the pseudopod appear in the plasmagel, especially near and in the region of attachment (Fig. 4). All this indicates marked strain, owing to violent contraction of the plasmagel especially in the proximal region of the pseudopod. This continues with increasing force until the attachment gives way. This sometimes takes place suddenly. If it does the pseudopod shortens so rapidly and so extensively, after the attachment has been broken, that the distal end actually snaps back to a point not more than half as far from the shell as it was. This demonstrates conclusively that the pseudopod was under rather violent strain before the attachment broke. Usually the attachment of the pseudopod breaks gradually. When this obtains, one point after another, here and there throughout the entire attached portion of the pseudopod, gives way and owing to this the regions of the plasmagel at the tip, which do not give way, are drawn out in strands of considerable length. Finally the attachment of these also breaks, but they retain their form for some time, giving the tip of the pseudopod a distinctly fibrous, brush-like appearance. These strands gradually retract, but the distal end of the pseudopod remains very irregular in outline and much flattened until it disappears (Fig. 4 *C*, *D*, *E*, *F*).

The facts that much of the substance in the attached portion of the pseudopod is drawn out in strands, that these strands contract rapidly and extensively after attachment to the substratum breaks, that the anterior surface of the pseudopod retains an irregular contour until it is withdrawn, and that the anterior end of the pseudopod remains much flattened after it is free, demonstrate conclusively that this substance is a fairly firm, highly elastic gel. These facts and others presented indicate that contact induces gelation, resulting in increase in thickness of the plasmagel throughout the entire length of the pseudopod, and that this causes increase in the elastic strength of the plasmagel, resulting in contraction in the pseudopod and expansion elsewhere. But why does the substance contract after it gelates? This is obviously the central problem concerning the processes involved. It is easy enough to understand why and how an elastic substance which has been stretched con-

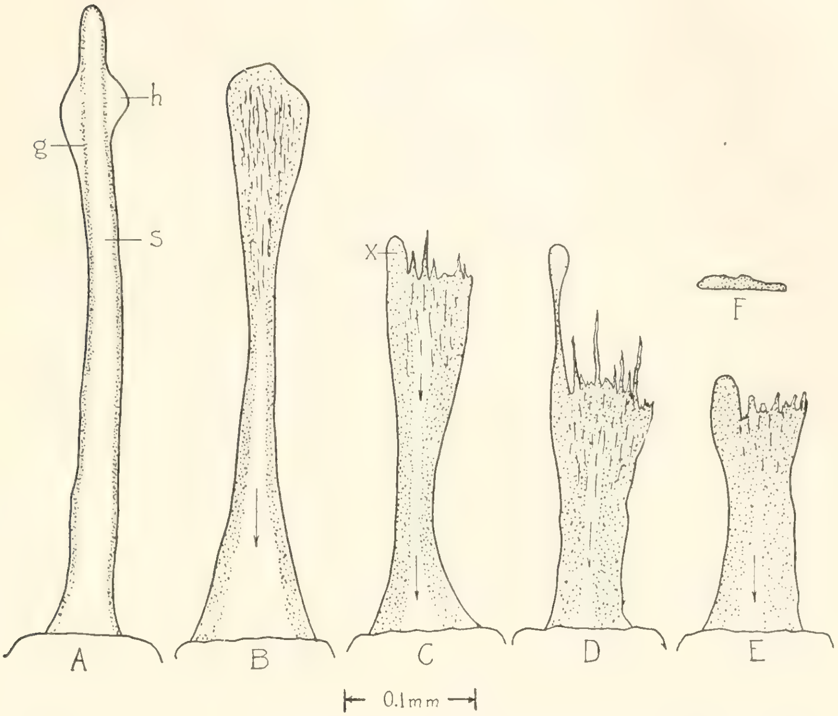

Fig. 4. A series of camera sketches of a pseudopod of *Difflugia pyriformis* representing different stages in the process of contraction in a specimen with the shell fastened to the substratum. *A,* early stage in the attachment of the pseudopod to the substratum; *B,* pseudopod firmly attached at the tip and beginning to contract elsewhere. Note that the plasmagel has increased in thickness and that it appears to be fibrous at the tip. *C, D,* and *E,* stages in the contraction of the pseudopod. Note that as the pseudopod contracts, and the attachment breaks, the cytoplasm at the tip of the pseudopod is drawn out into irregular strands which usually shorten considerably after they become detached, indicating that the cytoplasm here is viscous and highly elastic. This was particularly evident in the projection labeled *x.* The tip of this projection remained attached to the slide for some time after the rest of the tip of the pseudopod became free. This resulted in great stretching of the projection. Finally the attachment broke, whereupon the projection contracted very rapidly and extensively as indicated. *F,* outline representing the cross section of the pseudopod near the distal end. Note that the pseudopod was much flattened.

tracts but why and how a sol which has gelated and has not been stretched contracts is difficult to understand. I shall consider this problem presently.

Difflugia pyriformis without Shells

Difflugia without a shell is, as far as my experience goes, never found in nature. Sometimes it will, however, leave its shell if the shell is slightly broken, as the following observations indicate.

Six specimens varying considerably in size were mounted under a cover-glass supported and sealed with a small ridge of vaseline. Then the cover-glass was gently pressed down until the shells were broken, some very slightly, others considerably, after which observations were made from time to time for six days. During the first day there was practically no movement in any of the specimens in which the shell was considerably broken. They were much rounded and there was no indication of formation of pseudopods, but they did not disintegrate. Those in which the shell was only slightly broken behaved normally, *i.e.,* pseudopods extended, became attached and contracted precisely as they do in specimens with intact shells. On the second day pseudopods appeared from time to time on various surfaces in those with badly broken shells. These pseudopods were at first short, but later they extended practically as far as they do in normal specimens. The formation of these pseudopods was not related to the mouth of the shell. They apparently developed equally readily on all surfaces, extending here and there through crevices in the broken shell. They often appeared alternately on opposite sides, one extending while the other contracted.

Later in the day three of the specimens left the shells. One, however, carried with it the edge of the mouth of the shell in the form of a ring. Through this pseudopods extended, one after another, became attached at the tip and contracted, pulling the body along just as in normal specimens. No pseudopods formed elsewhere on the body. In this specimen it could be clearly seen that the plasmasol in the contracting pseudopod flowed directly into an extending pseudopod which developed from the base of the contracting pseudopod as a branch. There was, however, no such violent and rapid shortening of pseudopods as was sometimes seen in normal specimens, especially when stimulated, as will be shown later. The shell therefore seems to function in this. Moreover, the shell serves to coördinate movement in that it confines the formation of pseudopods to one region on the surface of the body; namely, that opposite the opening in the shell. This becomes evident if the movement of normal specimens or that of the specimen with the ring, just described, is compared with that of the specimens which were entirely naked.

In these specimens after they had left the shells just as before they had left it, the formation of pseudopods was not restricted to one surface. As a matter of fact, successive pseudopods rarely formed in the same region of the body; indeed they often formed on diametrically opposite surfaces. This resulted in movement, now in one direction and then in another; movement which in comparison with that of normal specimens was very irregular in direction and quite uncoördinated.

For some time after these specimens left the shells the pseudopods extended, attached and contracted, pulling the body along, much as they do in normal specimens. Sometimes two pseudopods appeared on opposite sides of the body simultaneously; then extended, attached and contracted, pulling the body out in opposite directions and greatly elongating it (Fig. 5). The following day all of the specimens were out of the shells and there were several small ones. These were probably fragments which had separated from the large ones. A large

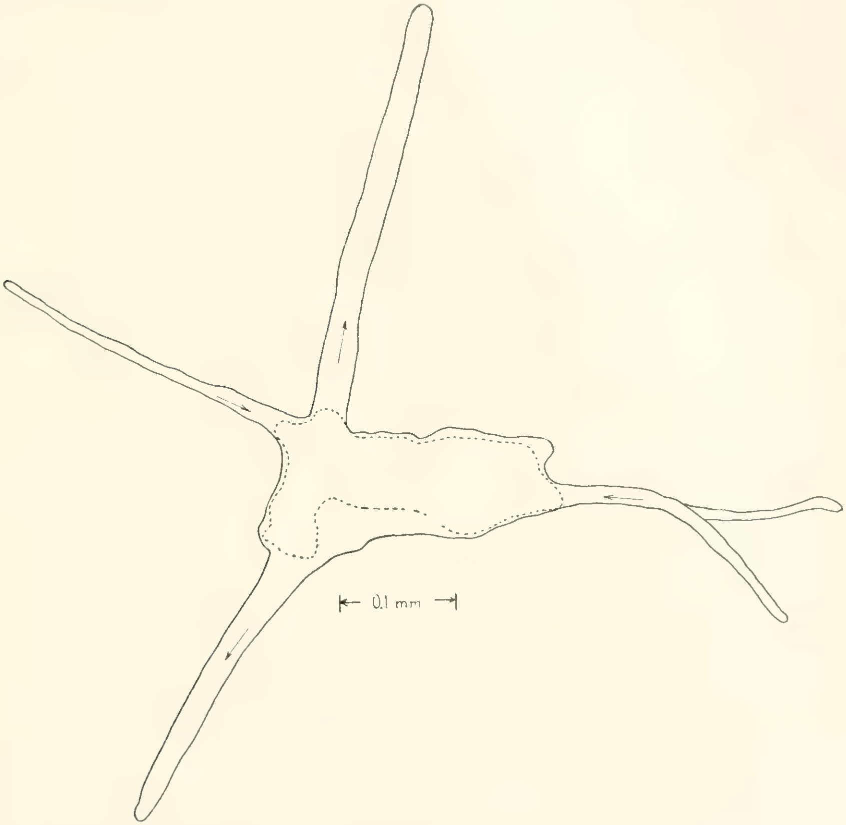

Fig. 5. Camera sketch of a specimen of *Difflugia pyriformis* two days after it had left its shell which had been broken by the pressure of the cover-glass. In this specimen two pseudopods which extended in opposite directions were several times seen to attach simultaneously and then to contract. This resulted in great elongation of the specimen. The portion enclosed by the broken line was well filled with chlorellae; the rest contained numerous small grayish granules.

granular nucleus and several contractile vacuoles could now be clearly seen in each of the large specimens (Fig. 6), but none was found in the small ones.

The small specimens moved about in a fairly coördinated fashion (Fig. 7) and some of the large ones now moved much more consistently in a given direction than they did on the preceding day, and in these the process of locomotion was in all essentials like that in *Amœba proteus* (Mast, 1926). Attachment of the pseudopods at the tip followed by contraction had practically disappeared. A pseudopod advanced in a given direction for a time, then stopped but did not contract. In the meantime another appeared near its base, advanced in the same general direction for a time and stopped, etc., just as in *Amœba proteus.*

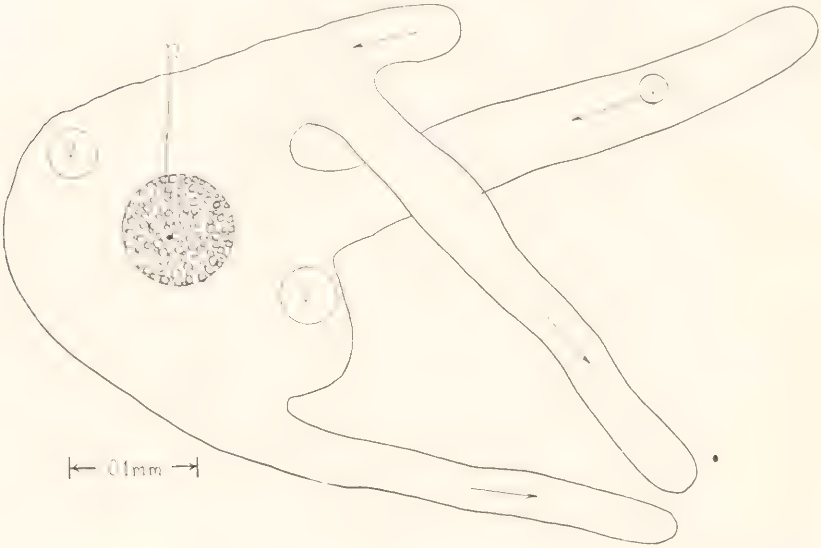

FIG. 6. Camera sketch of a specimen of *Difflugia pyriformis* several days after it had left its broken shell. *n,* nucleus; *v,* contractile vacuole; arrows, direction of flow in the plasmasol.

This specimen moved about fairly consistently and the process of locomotion was essentially the same as that in *Amœba proteus.* It contained many chlorellae, but they were scattered, making it possible to see the nucleus and the contractile vacuoles.

The movement of the plasmasol in the pseudopods could be very distinctly seen in these specimens, owing to the fact that the chlorellae were carried out in the pseudopods very much farther than they were in normal specimens. Sometimes they were carried to the tip of the pseudopods, but they never were caught in the gelation of plasmasol at the tip and consequently never became a part of the plasmagel. This resulted in a very definite differentiation between the plasmasol and the plasmagel, the former being green and the latter greyish.

The following day movement continued in the same way but the specimens were less active, and 24 hours later all of the specimens were much rounded and there was but little movement. All of the specimens lived a few days more, *i.e.,* they lived without shells nearly a week.

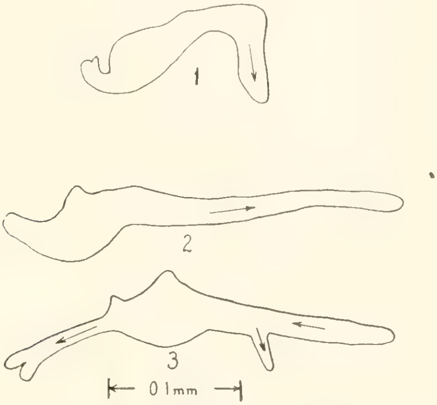

Fig. 7. Camera outlines of a fragment of *Difflugia pyriformis* produced by cutting off a pseudopod from a normal specimen. Interval between successive outlines, 2 minutes; arrows, direction of streaming.

Note that the fragment changed rapidly in form and that relatively large pseudopods developed. This fragment moved about freely and the process of locomotion was in full accord with that in *Amœba proteus.* The fragment contained a surface membrane (plasmalemma), a thin gel layer under this (plasmagel), and a central fluid mass (plasmasol).

These observations were repeated twice with essentially the same results. The fact that the process of locomotion in naked specimens of *Difflugia* is like that in *Amœba proteus* strongly supports the contention that in normal specimens the principles involved are fundamentally the same as in *Amœba.*

Difflugia acuminata

Difflugia acuminata is very much like *Difflugia pyriformis* in structure, the only marked difference being a protuberance on the base of the shell; but it is much smaller, the specimens studied being only about .17 mm. in length and .1 mm. in width.

The process of locomotion is essentially the same in the two species. In both, pseudopods one after another form, extend, attach and contract, pulling the shell along in steps. In both the plasmagel in the pseudopod is in the form of a tube open at the end, and the plasmasol flows out through this and gelates at the distal end, thus building the gel tube forward and extending the pseudopod. In both, when the pseudopod contracts, the plasmasol flows back through the plasmagel tube, but in *D. acuminata* the backward flow is much more regular than in *D. pyriformis* and it almost invariably flows directly into a new pseudopod which usually extends as the old one contracts (Fig. 8), while in *D. pyriformis* it usually flows back into the body and from there out into a new pseudopod.

Locomotion in *D. acuminata* is essentially in accord with the description of locomotion in *Difflugia sp.* presented in a preceding paper (Mast, 1926, p. 413). It resembles locomotion in *Amœba proteus* with the exception of the rather violent contraction of the pseudopod after attachment at the tip, resulting in marked periodicity in the rate of movement. There is, however, also a tendency toward periodicity in the rate of movement in *Amœba proteus* which was clearly demonstrated by Schwitalla (1924), and it may be that the processes involved in producing this periodicity are fundamentally the same in both forms, *i.e.*, that there is a certain amount of contraction in the pseudopod after attachment in *Amœba* as well as in *Difflugia*.

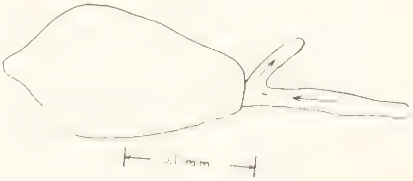

FIG. 8. Camera outline of *Difflugia acuminata*, illustrating the flow of plasmasol from the old pseudopod directly into the new one.

RESPONSE TO LIGHT

Difflugia pyriformis is definitely positive to light. It does not orient, at least not precisely, but in dishes left in front of a window it aggregates on the more highly illuminated side. This was repeatedly observed in the culture dishes, but it was more evident in a series of tests made by evenly distributing specimens in watch glasses in front of a north window, leaving them for a time and then ascertaining the distribution.

The results obtained in several tests are essentially the same. In one test consisting of five watch-glasses containing a total of 131 individuals there were at the end of 12 hours 103 in the window-half of the dishes and only 29 in the opposite half.

Concerning the process of aggregation I have no information. Rapid increase in illumination probably causes cessation in streaming, but this response if it actually does occur is far less definite than it is in *Amœba proteus*. In naked specimens it was observed that locomotion is much more rapid in low illumination than it is in high, but rapid increase in illumination did not result in sudden cessation in streaming.

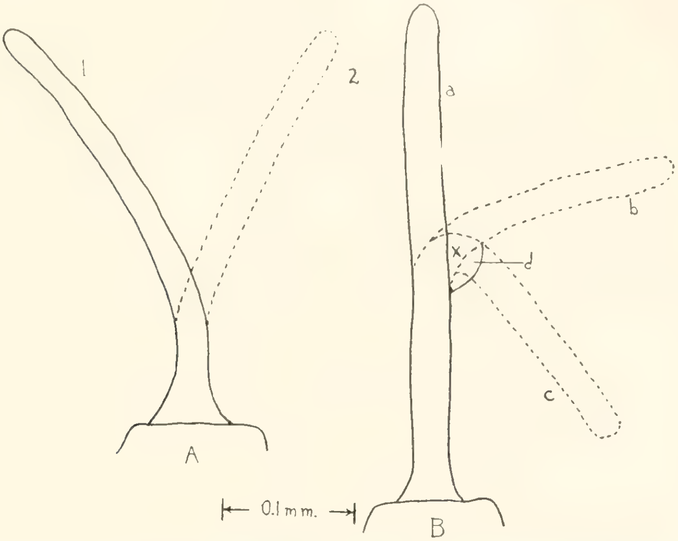

Fig. 9. Camera outline of *Difflugia pyriformis*, illustrating bending of pseudopods. *A*, bending without stimulation; *B*, bending after light mechanical stimulation at x; 1, original position of pseudopod; 2, position 1.5 seconds later; *a*, position of pseudopod before stimulation; *b* and *c*, positions one and two seconds later respectively; *d*, blister formed on the side of the pseudopod at the point stimulated.

Response to Contact

Response to contact was studied as follows: Several specimens of *Difflugia pyriformis* were put into a watch-glass under a dissecting binocular and left until they became active. Then with a glass needle pseudopods in different stages of development were touched in various ways and the effects noted. The results of numerous observations may be summarized as follows:

If the needle is gently brought into contact with the side of a pseudopod, the pseudopod at the point touched almost immediately bends sharply and fairly rapidly toward the side touched. The tip, in this response, frequently swings through more than ninety degrees in less than two seconds (Fig. 9). This bending is obviously due to contraction of the plasmagel in the region stimulated. Here, immediately after the needle comes in contact with the surface, there is formed a small blister. In a preceding section we have noted that the formation of blisters is associated with thickening of the plasmagel, *i.e.*, with gelation of plasmasol adjoining the plasmagel in the region where the blister appears. If this is true, it is evident that contact causes thickening of the plasmagel, owing to gelation of adjoining plasmasol and that this causes local increase in the elastic strength of the plasmagel and contraction in this region resulting in the bending of the pseudopod.

If, in place of bringing the needle gently in contact with one spot on the pseudopod, it is in close succession brought rather violently in contact with several spots fairly uniformly distributed over the surface, the entire pseudopod contracts, and this usually continues until the pseudopod has been drawn entirely into the shell. The contraction of the pseudopod is sometimes gradual, the pseudopod gradually receding into the shell, but it is usually very rapid, as a matter of fact, so rapid that the pseudopod jerks back into the shell much as a tubicolous annelid jerks into its tube when it is violently stimulated. Verworn (1889, 1914) obtained similar responses in *Difflugia urceolata*.

The withdrawal of the pseudopod is often retarded by a mass of cytoplasm in the mouth of the shell, from which the pseudopod projects. This mass of cytoplasm forms a sort of stopper; it fills the mouth of the shell and projects over the edge in the form of a flange (Fig. 1). When this obtains, the pseudopod, after it is stimulated, shortens, then holds its position until the flange gives way, after which the whole mass, pseudopod and all, suddenly darts into the shell.

The fact that this whole mass of cytoplasm is thus suddenly drawn into the shell shows that stimulation of the pseudopod causes marked contraction in that portion of the body which is located in the neck of the shell and that the body of the organism is fastened to the shell in the basal region; for if this portion of the body were not fastened, it would be drawn forward in place of the portion in the neck of the shell being drawn backward.

When a pseudopod, owing to stimulation, contracts as described above, numerous small hyaline blisters form on the surface. This, as previously demonstrated, is associated with thickening of the plasmagel, owing to gelation of the adjoining plasmasol, and this in turn produces increase in the elastic strength of the thickened plasmagel, resulting in contraction. The fact that stimulation of the pseudopod causes not only gelation and contraction of the region stimulated but also of certain portions of the body within the shell, *i.e.*, in a region some distance from the location of the stimulus, shows that localized contact stimulation produces in *Difflugia* something which is transmitted through the cytoplasm and then causes gelation of plasmasol followed by contraction. That is, it produces something which is akin to what in higher forms is known as an impulse. This conclusion is supported by results obtained by Verworn in observations on the effect of localized mechanical stimulation of *Difflugia urceolata*. He (1889, 1914) maintains that local stimulation of a pseudopod causes, under certain conditions, contraction of pseudopods which were not stimulated. There is, however, no evidence indicating that this obtains for all rhizopods. Verworn was not able to find it in other species of *Difflugia* and it probably does not occur in *Amœba* (Mast, 1932).

Discussion

I have demonstrated in the preceding pages that the more important factors involved in locomotion and response in *Difflugia* consist of solation of the plasmagel in one region of the body and gelation of the plasmasol in another, of attachment of the tip of the pseudopod to the substratum, of gelation of the plasmasol in the pseudopod correlated with contact and of contraction of the pseudopod due to increase in the thickness and in the elastic strength of the plasmagel in it.

All of these factors are probably also involved in the process of locomotion and response in *Amœba,* but gelation associated with contact followed by contraction of the pseudopod, which plays such an important rôle in the process of locomotion in *Difflugia,* is of little if any significance in the process of locomotion in *Amœba*. To account for locomotion in *Difflugia,* it is necessary then to explain not only gelation and solation but also contraction of the substance after it has gelated. Concerning the processes involved in this, I have no suggestions to offer except that the fact that fluid is squeezed out during gelation and contraction indicates that the processes involved are in some respects similar to those associated with syneresis as found in the gelation of various inanimate substances.

Summary

1. The fleshy part of *Difflugia pyriformis* and *Difflugia acuminata* is in structure much like *Amœba proteus*. There is a thin elastic surface membrane (plasmalemma), a central fluid mass (plasmasol), containing a large granular nucleus, and a relatively solid layer (plasmagel) which surrounds the plasmasol. Probably there is also a hyaline fluid layer between the plasmagel and the plasmalemma but this, if it is present, is much thinner and less conspicuous than in *Amœba*.

2. Locomotion in *Difflugia* is normally brought about by the extension of pseudopods, one after another, and attachment to the sub-

stratum at the tip, followed by contraction which pulls the shell containing the body forward. Movement is consequently intermittent.

3. The elastic strength of the plasmagel is lowest at the tip of the pseudopods. This results in contraction of the plasmagel elsewhere, and this contraction forces the plasmasol out through the plasmagel tube, causing expansion at the tip of the pseudopod. The plasmasol which is in contact with the distal edge of the plasmagel tube continuously gelates and this results in extension of the tube.

4. After the tip of the pseudopod becomes attached, the plasmasol in the tip gelates and the plasmagel throughout the entire pseudopod thickens greatly owing to gelation of adjoining plasmasol. This increases the elastic strength of the plasmagel in the entire pseudopod until it becomes greater here than elsewhere, after which it contracts and the pseudopod becomes shorter and thicker and the plasmasol in it is forced back into the body of the organism.

5. The extension of pseudopods in *Difflugia* is in principle the same as in *Amœba proteus*, and contraction is probably also the same in principle, but it is much more pronounced and much more highly specialized in *Difflugia* than in *Amœba*, in which it does not function appreciably in the process of locomotion.

6. The pseudopods in *Difflugia* after they are extended wave about considerably. This is doubtless due to unequal local contraction of the plasmagel on opposite sides.

7. If the shell of *Difflugia pyriformis* is broken, it leaves the shell in the course of a day or so and moves about fairly freely. Specimens out of the shell sometimes live for a week or more under a cover-glass supported and sealed with a ridge of vaseline. After they have been out of the shell for some time the process of locomotion in such specimens is in all respects like that in *Amœba*, although it is usually much more irregular in direction. Contraction of extended pseudopods, so conspicuous in the process of locomotion in specimens containing shells, has practically disappeared.

8. *Difflugia pyriformis* aggregates on the more illuminated side of dishes in moderate illumination. It does not orient precisely. It is less active in high illumination than in low, especially when it is out of the shell. It may be that this functions in the aggregation observed. Rapid increase in illumination probably causes decrease in the rate of streaming, but this, if it occurs, is much less marked than it is in *Amœba proteus*.

9. Weak local contact stimulation of an extended pseudopod causes sharp bending in this region toward the side stimulated. Strong general contact stimulation causes rapid contraction of the entire pseudopod.

The bending is due to local thickening of the plasmagel in the region stimulated. Contraction of the entire pseudopod is due to thickening of the plasmagel in the entire pseudopod.

10. Contact stimulation results, under certain conditions, in gelation which extends far beyond the region stimulated. There is therefore in *Difflugia* transmission of something akin to an impulse in higher forms.

LITERATURE CITED

Mast, S. O., 1926. Structure, Movement, Locomotion and Stimulation in Amœba. *Jour. Morph. and Physiol.*, **41**: 347–425.

Mast, S. O., 1932. Localized Stimulation, Transmission of Impulses and the Nature of Response in Amœba. *Physiol. Zoöl.,* **5,** in press.

Verworn, Max, 1889. Psycho-phsiologische Protisten-studien. Jena, 218 S.

Verworn, Max, 1914. Erregung und Lähmung. Jena.

THE EFFECT OF DILUTION OF SEA WATER ON THE ACTIVITY AND LONGEVITY OF CERTAIN MARINE CERCARIÆ, WITH DESCRIPTIONS OF TWO NEW SPECIES

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INTRODUCTION

The present investigation was undertaken to secure data bearing on the question of the origin, distribution, and evolution of present groups of digenetic trematodes. The specific problem under consideration develops from the observation that several families of the digenetic trematodes have representatives in both marine and fresh-water hosts. A brief statement of the problem was given by Stunkard (1930). If the trematodes found in both marine and fresh-water hosts and assigned to common families, and even to common genera, have true phylogenetic relationships; *i.e.,* if they have descended from common ancestors, rather than consisting of groups that through convergence show morphological and developmental similarities, their distribution raises an exceedingly difficult biological problem.

There appears to be little doubt but that the parasites in question are actually closely related. Among the gasterostomes, *Bucephalus polymorphus* von Baer, 1826 was described from fresh-water fishes and *Bucephalus haimeanus* Lacaze-Duthiers, 1854 was described from marine fishes. Tennent (1906) traced the life cycle of the latter species, and the recent studies of Woodhead (1929, 1930) have demonstrated the development of two species that occur in fresh-water hosts. The similarity in structure and development between the marine and fresh-water species is so striking that it strongly indicates close relationship.

In the Prosostomata there are a number of families whose members occur in both marine and fresh-water hosts. The family Aspidogastridæ, *e.g.* (see account by Stunkard, 1917), contains species that infest mollusks, fishes and turtles of fresh water, and others that occur abundantly in marine fishes. In this aberrant family, also, the morphological and developmental agreement is too close to be satisfactorily explained on the basis of convergence.

Several other families of the Digenea manifest the same type of distribution. The family Fasciolidæ (see Stunkard and Alvey, 1930) contains one group of genera which infests the livers of terrestrial herbivores, and another, consisting of *Campula, Lecithodesmus, Orthosplanchnus,* and *Zalophotrema,* which occurs in the livers of various marine mammals. In the family Paramphistomidæ there are a large number of genera, most of which parasitize the hoofed mammals, although one genus, *Chiorchis* (see Stunkard, 1929) occurs in the Atlantic manatees. It is of course possible that the sea cows, frequenting the mouths of rivers, acquire these parasites in fresh water. With few exceptions, members of the Heterophyidæ occur in terrestrial vertebrates and the larvæ develop in fresh-water snails, while *Cryptocotyle lingua* infests the intestine of fish-eating birds and the larvæ develop in the marine snail *Littorina littorea* (see Stunkard, 1930*a*). In the family Pronocephalidæ, several genera have been reported from marine turtles, while one species was described (Stunkard, 1930) from the fresh-water turtle, *Amyda,* and a second has been described by Mackin (1930), from *Pseudemys elegans.* According to Fuhrmann (1928) the family Steringophoridæ (syn. Fellodistomidæ, see Stunkard and Nigrelli, 1930) contains species from the intestine of both marine and fresh-water fishes. Representatives of all of these groups have been studied by the senior author and the results afford cumulative evidence that both marine and fresh-water hosts harbor closely related species of parasites.

Nicoll (1915, 1924) lists other families which have representatives in both marine and fresh-water fishes. Furthermore, there are several genera, *e.g., Azygia* (see Manter, 1926), which have species in both marine and fresh-water hosts. No exhaustive review of the literature is here attempted, but sufficient data have been presented to indicate that many groups of trematodes which manifest marked similarity in structure and development have members, some of which infest marine and others which infest fresh-water hosts. The agreement in morphology and manner of development, recurring so consistently in different groups, can hardly be fortuitous, and the majority of investigators are agreed that these groups are formed by closely related rather than convergent species.

If, as has been postulated, these groups contain closely related species, the question naturally arises as to whether the common ancestral form occurred in marine or fresh-water hosts and how the present distribution was effected. A factor which must be kept in mind throughout the discussion is the complicated life history of these digenetic forms. Typically, sexual multiplication occurs in a vertebrate host, and eggs are produced which pass from the body of the host. From the eggs there emerge aquatic, ciliated larvæ which invade the first intermediate host, always an invertebrate and usually a mollusk, where asexual multiplication takes place. A second type of aquatic, tailed larvæ leaves the first intermediate host, and these larvæ, either by direct penetration or after encystment on aquatic plants or in the bodies of other intermediate hosts, finally reach the vertebrate host. The free-living, larval stages are extremely delicate, ephemeral, and incapable of any extended migration. Consequently the presence of members of a common group in both marine and fresh-water hosts can only be explained by migration of the hosts or by transfer to new hosts.

The migration of free-living species from a marine to a fresh-water habitat, or the reverse, is largely prevented by the physical, chemical, and biological factors that characterize the two types of environment. It is true that marine species have been cut off in arms of the ocean, *e.g.,* the Caspian and Black Seas, which have subsequently become bodies of fresh water, and some of them have persisted although the number of such species is not large. Among the fishes, the anadromous and catadromous forms make regular migrations from one habitat to the other, but these examples stand as exceptions to the general rule. Fresh water imposes an effective barrier against the migration of Foraminifera, corals, echinoderms, cephalopods, and other groups of invertebrates. The relatively few invertebrates that have transferred from the ocean to fresh water have undergone extensive modifications in form and in life history. The free-swimming larval stages, characteristic of marine types, have almost entirely disappeared.

In an excellent study of this subject Needham (1930) has discussed the factors which prevent the penetration of marine organisms into fresh water. Allee (1923) has shown the effect of differences in temperature, oxygen content, and hydrogen ion concentration on the distribution of littoral invertebrates. Adolph (1925) studied certain physiological distinctions between fresh-water and marine organisms. He found that marine organisms show a much greater toleration for fresh water than fresh-water organisms do for sea water. His observations tend to support the long-accepted belief that organisms migrate from the ocean into fresh water, rather than in the opposite direction. In an interesting and suggestive study Marshall and Smith (1930) have attempted to correlate the composition of the body fluids of marine and fresh-water fishes with renal function and to trace the evolution and migration of these vertebrates on the basis of changes in the structure and activity of the excretory organs. Pantin (1931) has studied the triclad turbellarian *Gunda ulvæ*, which occurs in the estuaries of small streams, and found that these acœlomate worms withstand both fresh and salt water, and that in nature they may be exposed to either extreme for several hours. In tap water they double their volume and lose 25 per cent of their salt content in an hour. The presence of calcium reduces the rate of swelling and the loss of salts, presumably by reducing permeability. The significance of calcium and its relation to the problem of the migration of animals into fresh water was discussed.

The difficulties of migration from one habitat to the other would be greater in the case of parasitic species like the digenetic trematodes than in free-living forms. Where two or more hosts are involved, and where the transfer to the next host is effected by very delicate, short-lived, larval stages, the initial obstacles to migration are augmented by the difficulties inherent in the completion of the life cycle. For such a parasite to change from one location to another, either both primary and secondary hosts must have made the same migration simultaneously, or the parasite must have changed to new hosts as the migration progressed. Furthermore, and probably of greatest importance, the free-living, aquatic, larval stages must be able to withstand the changed environmental conditions and remain infective.

The migration of primary and secondary hosts has not been extensive in recent times at least. The groups of mollusks, fishes, reptiles, and mammals are clearly separated into marine and fresh-water species and this distinction has persisted with but very little change since earlier geological time. The shells of mollusks and skeletons of vertebrates afford suitable material for fossil formation and the geological history of several of these groups is known. According to Zittel (1913) "By means of analogy with recent species we are able in most cases readily to determine whether fossil forms pertain to land, fresh, brackish, or salt-water species." He stated that "not until the boundary between the Jura and Cretaceous is reached do we find any traces of fresh-water snails. . . . In the Wealden, and Cretaceous generally, both land and fresh-water gastropods are quite abundant; they become highly developed and widely distributed during the Tertiary, attaining, in fact, a differentiation nearly equal to that exhibited by the corresponding recent forms."

Since the vertebrate hosts are more active, wider ranging, and longer lived than the molluscan hosts, it would appear probable that if migration is to be considered as the explanation of present distribution, the vertebrate hosts were the principal migrants and that they were primarily responsible for change of habitat. The paleontology of the turtles (Hay, 1908; Williston, 1914) indicates that the marine turtles and the soft-shelled, fresh-water turtles have been separate, independent groups since the Mesozoic era. Looss (1902) described several genera of pronocephalid trematodes from marine turtles; Stunkard (1930) and Mackin (1930) have described members of the same family from the fresh-water turtles, *Amyda* and *Pseudemys*. The discovery of related parasites in hosts that have been separated since the Mesozoic would suggest that migration of hosts is not to be accepted as an explanation of these cases at least.

There are also serious objections to the explanation involving transfer to new hosts. While host parasite specificity is not so limited as was formerly believed, and it is well known that many parasites may infest several host species, as a rule the possible host species are closely related. For this hypothesis it is essential also that both old and new hosts live in the same habitat, since otherwise they would never encounter the infective larval stages of the parasite. Consequently, if separation into marine and fresh-water species was effected by the adoption of new hosts, the transfer could occur only in those regions where fresh and salt-water habitats overlap, namely at the mouths of rivers. In the transitional zone of brackish water, with the recurrent increase and decrease of the salt content and pH of the water with the rise and fall of the tide, transfer to new hosts may have caused divergence into definitely marine and fresh-water species.

It is thus possible that both migration of hosts and transfer to new hosts or a combination of the two methods may have been operative in producing present distribution of related species. It may be that the distribution of existing groups of digenetic trematodes is correlated with the origin of these groups and this point should be considered in any treatment of the problem. The present complicated developmental cycles could not have been the original or primitive life histories of these species. It has long been recognized that parasites have been derived from free-living progenitors. Competent investigators agree that the trematodes and cestodes have a turbellarian ancestry. The subject was discussed by Meixner (1926) with the following summary, " Es ergeben sich drei Schlüsse:

" I. Dass die Differenzierung der Trematoden und Cestoden mit dem Auftreten der Wirbeltiere eng verknüpft ist.

" II. Dass die zum Parasitismus auf Evertebraten übergegangenen Vorfahren der Digenea und Cestoden entsprechend der heutigen Beschränkung der primären Larven bereits auf verschiedene Wirtstierklassen spezialisiert waren.

" III. Trematoden und Cestoden sind zwei infolge ihres Parasitierens auf Wirbeltieren insbesonders hinsichtlich des Integumentes der Reifestadien abgeanderte Anhangsgruppen der Rhabdocoela."

Concerning the origin of these groups Reisinger (1928) stated, " Bezeichnend für die Amera ist die in vielen Gruppen vorherrschende Neigung zu parasitärer Lebensweise, vielleicht in Ausnützung einer besonderen, dem ganzen Unterstamm eigenen, stoffwechselphysiologischen Konstitution, die den einzelnen Gruppen den Ubergang zu intramolekularer Atmung (Glykogenabbau) besonders erleichterte. Die Urheimat der Amera ist zweifellos das Meer; der Ubergang zu terrikoler und parasitischer Lebensweise mag sowohl von dort aus wie auch vom Süsswasser erfolgt sein und erfolgen."

Bresslau and Reisinger (1928) concluded that, "Unter den Rhabdocoelen verdienen die Familien der Graffilliden und Anoplodiiden besonderes Interesse, insofern als von ihnen aus vermutlich die Entwickelung der Trematoden ihren Ausgang genommen hat. Nach ihrer ganzen Organization sind die Monogenea wahrscheinlich von Graffilliden, die Digenea von Anoplodiiden oder anoplodiidenähnlichen Kalyptorhynchiern (Rhabdocoela) herzuleiten. Gut stimmt damit überein, dass gerade diese Familien das Hauptkontingent an Parasiten unter den Strudelwürmer stellen."

Consideration of this subject raises one of the most difficult problems in biology, the origin of intermediate hosts and the digenetic life cycle. The original ancestors of the digenetic trematodes must have become parasites of aquatic animals and the evidence indicates that mollusks were the original hosts. The type of reproduction in these mollusks is problematical. It is well established that parasitism increases reproductive activity, that it leads to new and accessory methods of reproduction, and that asexual multiplication is frequently interpolated between the sexual phases. There may have been a sexually mature, free-living stage after asexual multiplication was developed in the invertebrate host. The appearance of the vertebrate host may be correlated with the evolution of vertebrates and their use of mollusks as food.

Presumably the adoption of the parasitic habit occurred at an extremely remote period and the evolution of parasitic life histories and accompanying transformation of the parasites have proceeded hand in hand with the evolution of their hosts. The parallel evolution of hosts and parasites has been demonstrated by many authors. The presence of related species in both marine and fresh-water hosts may be explained by assuming that the primitive hosts harbored the ancestors of present species, and that the hosts have subsequently separated and differentiated into marine and fresh-water species. Such an explanation would imply that descendants of the original hosts have carried their parasites with them since the separation and, as a result of the ensuing migration and modification, the primary hosts, secondary hosts, and parasitic species have evolved together. It may be contended that this explanation merely pushes the problem further back in the time scale, renders it more difficult of analysis, and less susceptible of experimental treatment. While to a degree this criticism is valid, the postulate may nevertheless be correct, and there are, moreover, experimental means of investigating the question.

Studies of much modified parasitic species and interpretation of their life histories are greatly facilitated if the life cycle contains free-living larval stages. These stages presumably correspond to ancestral ones, since it is generally true that embryonic and early developmental stages are very conservative and tend to remain unchanged regardless of modifications which may occur in the later development of the animal concerned. Since digenetic trematodes have such free-living larval stages, and since these larvæ are the infective agents, providing for the transfer from one host to another,—an experimental study is possible. Knowledge concerning the effects of environmental changes on these larvæ may have significant value in the interpretation of life cycles and distribution. Since the trematodes have two free-living larval stages in the life cycle, data should be obtained for both the miracidial and cercarial stages. It is often difficult if not impossible to secure miracidia in sufficient numbers for such experiments, while cercariæ can usually be obtained in abundance. It therefore seemed pertinent to make a study of the effect of the dilution of sea water on the activity and longevity of marine cercariæ. A corresponding study, already started, on the effects of diluted sea water on fresh-water cercariæ will give data, which, correlated with those from the present investigation, may aid materially in explaining present distribution of related species in marine and fresh-water hosts.

No matter whether the present distribution is explained through migration of original hosts or transfer to new hosts, the essential factor involved is the ability of the free-swimming larvæ to live and remain infective in the new environment. The ability of these larval stages to function in increasing or decreasing salinity indicates the direction of migration and the original home of the original trematode species. Consequently, the experiments reported in the present paper were undertaken.

Material and Methods

All of the cercariæ used in the investigation were obtained from mollusks of the Woods Hole region, and the experiments were done at the Marine Biological Laboratory during the summer of 1930. Data are given in the tables for the following six species: (1) the cercaria of *Cryptocotyle lingua* from *Littorina littorea*, (2) *Cercariæum lintoni* from *Nassa obsoleta*, (3) *Cercaria quissetensis* from *N. obsoleta*, (4) *C. variglandis* from *N. obsoleta*, (5) *C. parvicaudata* from *L. littorea*, and (6) *C. sensifera* from *Urosalpinx cinereus*.

The snails were isolated in small dishes of sea water to determine those from which cercariæ were emerging. Several of those infected by one and the same species were then placed in a small dish for 10 to 12 hours in order to secure large numbers of recently emerged cercariæ. At the end of this period the snails were removed and the cercariæ transferred to small dishes of sea water, usually 20 to 50 in each dish. The sea water was removed from these dishes and replaced by sea water to which various amounts of tap water had been added. Solutions were made up as follows: (I) undiluted sea water; (II) ¾ sea water, ¼ tap water; (III) ½ sea water, ½ tap water; (IV) ¼ sea water, ¾ tap water; (V) ⅛ sea water, ⅞ tap water; (VI) tap water. The dishes were covered to prevent evaporation and kept at the temperature of the laboratory. In each experiment all of the larvæ were subjected to identical conditions except for the different amounts of tap water in the solutions. The only variable factor, therefore, was the amount of tap water and the results show the effects of increasing dilutions of sea water. Observations were made with a binocular microscope at appropriate intervals and the condition and activity of the larvæ noted.

Cercaria of Cryptocotyle lingua

An abstract of this experiment was reported (Stunkard, 1930*c*).

At first the larvæ are very active and all swim vigorously by rapid lashing of their tails, holding the body motionless in a curved position. Swimming movements cause the cercariæ to rise toward the surface of the water and when swimming is temporarily suspended the larvæ slowly sink. They are positively phototropic and accumulate at the light side of the dish. As the vitality of the larvæ diminishes they become progressively weaker and are unable to rise from the bottom. This is due primarily to exhaustion of the tail muscles. The larvæ then extend and retract their bodies and tails, although since there is no functional acetabulum they can make little forward progress. In the solutions which contain 50 per cent or more of tap water the tails soon begin to swell and lose their motility, and later the body swells. Naturally the swelling is more rapid and greater in the more dilute solutions. Apparently swelling is inhibited so long as the tissues are alive and active. The swelling causes cytolysis of the tails and they soon drop off. The tails are frequently lost after a few hours in all of the solutions. In the tables the following notations are used:

Swimming vigorously ++++
Swimming seldom and feebly +++
Contracting vigorously ++
Contracting feebly +
Dead —

The experiments were repeated four times and the results are in substantial agreement. The following protocol is representative.

Solutions	I	II	III	IV	V	VI
No. of cercariae Aug. 4 Time	25	69	53	70	140	172
2:00 P.M.	25	69	53	70	140	172
2:20	all + + + +	all + + + +	all + + + +	all + + + +	all + + + +	80 + + 92 +
2:30	all + + + +	all + + + +	all + + + +	all + + + +	all + + + +	86 + 86 −
3:00	all + + + +	all + + + +	all + + + +	68 + + + + 2 + + +	50 + + + + 90 + + +	1 + 171 −
4:00	all + + + +	all + + + +	all + + + +	65 + + + + 4 + + + 1 + +	140 + +	
6:00	23 + + + + 1 + + + 1 + +	60 + + + + 5 + + + 2 + + 2 −	44 + + + + 7 + + + 1 + + 1 −	58 + + + + 11 + + + 1 + +	6 + + + + 4 + + + 32 + + 10 + 88 −	

Solutions	I	II	III	IV	V	VI
No. of cercariæ Aug. 4 Time	25	69	53	70	140	172
8:00 P. M.	19++++ 3+++ 3++	57++++ 8+++ 2+ 2−	33++++ 18+++ 1++ 1−	56++++ 13+++ 1++	7+++ 35++ or + 97−	
Aug. 5 9:00	8++++ 15+++ 1++ 1−	53++++ 10+++ 6−	15++++ 31+++ 6++ 1−	21++++ 30+++ 7++ 5−	10+ 130−	
11:15	8++++ 14++ 3−	41++++ 20++ 8−	14+++ 36++ 3−	15+++ 50++ 5−	5+ 135−	
1:15	22++ 3−	35++++ 26++ or + 8−	50++ or + 3−	5+++ 60 ++or + 5−	5+ 135−	
3:15	22++ 3−	25+++ or ++ 36++ or + 8−	50++ or + 3−	5+++ 60++ or + 5−	2+ 138−	
Aug. 6 9:00	4+ 21−	5+ 64−	53−	70−	140−	

Analysis of the data shows that Solutions II, III, and IV have only slightly harmful effects as the sea water is diluted. In solutions containing 50 per cent or more of sea water the effect is not significant and, in two of the experiments, after 12 hours in Solution No. II the cercariæ were more active and vigorous than those in undiluted sea water. In Solution No. V, which contained 1/8 sea water, the cercariæ were all on the bottom of the container at the end of 2 hours. They had begun to swell noticeably, some had lost their tails, and those whose tails beat rapidly were unable to rise in the water. It is probable that the larvæ are not infective in this concentration for more than a few minutes. The range between 1/8 and 1/4 sea water appears to be the critical zone where the dilution of the sea water exerts a markedly harmful effect on the physiological processes of the larvæ. Freshly emerged cercariæ manifest normal swimming movements for only a few minutes when placed in tap water; at the end of 20 minutes all had settled to the bottom, in 30 minutes about 1/2 of them showed no sign of life and the others soon succumbed.

Cercariæum lintoni Miller and Northrup, 1926

Solutions were made up as in the previous experiment and the same procedure was followed. Since these larva have no tails they can not swim and their activity is restricted to creeping movements as described by Miller and Northup (1926). The experiment was repeated seven times using 20 recently emerged cercariæ in each dish. In two of the tests distilled water was used instead of tap water and the larvæ lived as long, and in certain of the dishes slightly longer than in those containing the same amount of sea water diluted with tap water. The differences were not great and probably are not significant. The results are similar for all experiments and the following protocol, given on p. 253, is typical.

These results are similar to those obtained for the cercariæ of *C. lingua*. The larvæ show very little normal activity after 15 minutes in tap water and it is apparent that they are not infective in this medium. Two larvæ encysted on the bottom of the dish but it is apparent that encystment in the water is not a usual or normal stage in the life history of the species.

Cercaria quissetensis Miller and Northup, 1926

The experiments were conducted as previously described and repeated eight times, using 20 cercariæ in each dish. In swimming, the

Solutions...............	I	II	III	IV	V	VI
No. of cercariæ..........	20	20	20	20	20	20
Time in Hours						
1	20++	20++	20++	20++	20++ or +	20−
2½	20++	20++	20++	20++	20++ or +	
6	20++	20++	20++	20++	18++ or + 2−	
12	20++	20++	20++	20++	13+ 7−	
24	17++ or + 3+ or −	16++ or + 4+ or −	16++ or + 4+ or −	15++ or + 5+ or −	12+ 8+ or −	
36	15++ or + 5+ or −	14++ or + 6+ or −	15++ or + 5+ or −	12++ or + 8+ or −	7+ 13+ or −	
48	11++ or + 9−	5+ 15+ or −	4++ or + 16+ or −	4++ or + 16+ or −	1+ 19−	
60	3+ 17−	2+ 18+ or −	2+ 18+ or −	1+ 19+ or −	20−	
70	1+ 19−	20−	1+ 2+ or − 17−	1+ 2+ or − 17−	20−	

body assumes a spherical form and the tail lashes vigorously. Records were taken every hour and the following protocol, given on pp. 255 and 256, tabulates the results of one experiment.

In this species the cercariæ lose motility after 15 to 30 minutes in tap water. The tails swell in all the solutions containing 50 per cent or more of tap water and soon become detached. There is a tendency for the cercariæ to encyst after 24 hours in the more concentrated solutions and such larvæ removed from their cysts at the end of 72 hours were alive and active. There were only slight differences between the cercariæ placed in Solutions I and II. During the first half of the experiment the larvæ in Solutions III and IV appeared to be affected more than those in Solutions I and II, but in three of the tests they lived longer than those in undiluted or 75 per cent sea water.

Cercaria variglandis Miller and Northup, 1926

This species is very rare. Miller and Northup found only 3 infested snails among 8,875 individuals of *Nassa* examined, and we found only 2 infected snails. The structure of the cercaria indicates that it is the larva of one of the blood flukes, and it swims in active spurts. Only two experiments were made, but the results, given in the protocol on p. 257, indicate that the larvæ are short-lived and very delicate. The procedure was the same as that previously employed.

As the cercariæ lose motility they become distorted, the furcæ coil up and it is sometimes difficult to determine whether or not they are dead. Soon, however, they turn dark-colored and later they tend to float.

Cercaria parvicaudata n.sp.

Two experiments were made with these larvæ. The procedure was the same as that previously employed, although the observations were not continued until the death of the cercariæ. One of the protocols is given on p. 258 and the other is in essential agreement.

The cercariæ in Solution VI (tap water) were all dead and their bodies much swollen at the end of one hour. At this time all of those in Solutions I, II, and III were swimming intermittently, while those in Solutions IV and V were unable to leave the bottom. They were all lying on the dorsal side, bodies bent as in swimming, with the tails moving. After four or five hours they seemed to be more active although they were unable to leave the bottom of the container. At the end of 25 hours, although they were unable to swim, the larvæ in Solution II were more active than those in sea water, and those in 50 per cent sea water were more active than those in Solution II.

Solutions	I	II	III	IV	V	VI
No. of cercariæ	20	20	20	20	20	20
Time in Hours						
1	20++++	20++++	20++++	20++++ or +++	20++++ or +++	20−
2	20++++	20++++	20++++	20++++ or +++	3+++ 17++	
4	20++++	20++++	20++++	20++++ or +++	2+++ 18++	
6	20++++	20++++	20++++	18++++ or +++ 2++ (tailless)	2+++ 18++ 8 tailless	
12	19++++ or +++ 1++ (tailless)	18++++ or +++ 2++ (tailless)	8+++ or ++ 12++ 9 tailless	7+++ or ++ 13++ 11 tailless	2+++ 18++ or + 15 tailless	
24	12+++ or ++ 7++ 1 cyst 8 tailless	8+++ or ++ 10++ 2−	4+++ or ++ 13++ or + 2 cysts 1−	5+++ or ++ 15++ or +	14++ or + 6−	

Solutions	I	II	III	IV	V	VI
No. of cercariae	20	20	20	20	20	20
Time in Hours						
36	7++ or + 2 cysts 6+ or − 5−	5++ or + 9+ or − 6−	13++ or + 2 cysts 5−	11++ or + 3+ or − 6−	8++ or + 3+ or − 9−	
48	5++ 2 cysts 13−	6++ or + 14−	7++ or + 2+ or − 9− 2 cysts	6++ or + 8+ or − 8−	5++ or + 3+ or − 12−	
60	2+ or − 2 cysts 16−	2+ or − 18−	3+ 2 cysts 15−	4+ 1 cyst 15−	20−	
70	2 cysts 18−	20−	2 cysts 18−	1+ 1 cyst 18−		

Solutions..........	I	II	III	IV	V	VI
No. of cercariæ.....	20	20	20	20	20	20
Time in Hours						
1	20++++	20++++	20++++	19++++ 1++	15++++ or +++ 5++	12+ 8−
2	20++++	20++++	19++++ or +++ 1++	17++++ or +++ 3+++ or ++	12+++ 8++	6+ 14−
6	14++++ or +++ 6+++ or ++	17++++ or +++ 3++	15++++ or +++ 5++	11+++ 4++ 5−	6+ 14−	20−
12	4+++ 12++ 4−	7++ 5+ 8−	5++ 3+ 12−	4++ or + 6+ or − 10−	20−	
24	20−	20−	2+ or − 18−	2+ 18−		

Solutions	I	II	III	IV	V	VI
No. of cercaria	39	65	60	80	90	100
Time in Hours						
1	39 + + + +	65 + + + +	60 + + + +	80 + +	90 + +	100 −
6	39 + + + +	65 + + + +	60 + + + +	80 + +	90 + +	
	39 + + + + or + + +	65 + + + + or + + +	60 + + + + or + + +	80 + +	38 + + 45 + 7 −	
25	32 + + or + 7 −	65 + + or +	59 + + or + 1 −	15 + + 55 +	15 + + 47 + 28 −	

Cercaria sensifera n.sp.

Four experiments were performed with these larvæ, and the results of one experiment are tabulated in the following protocol, given on p. 260.

There is a pronounced tendency for these cercariæ to encyst when subjected to unfavorable conditions. The process of encystment is rapid and the cysts are either free or attached to the bottom of the container. Presumably this phenomenon is normal and significant for life history studies of the species.

Cercariæ emerge usually at night or in the early morning and the majority soon encyst. They tend to adhere to any object they touch and numbers stick to the inside of a pipette used to transfer them from one solution to another. After attachment they soon encyst.

Discussion

In an investigation of this character, it is desirable to study as many species as possible and representatives of different taxonomic groups. Unfortunately, information concerning the marine larval trematodes of North America is very meager. Only a random sample of the species has been described. The literature dealing with these larvæ was reviewed by Miller and Northup (1926), who described five species from *Nassa obsoleta* at Woods Hole, Massachusetts. It is significant that only one of the five had previously been reported. Since these were almost the only larval trematodes described from the Woods Hole region, an attempt was made to secure them for the present study. All of the five species described by Miller and Northup were found and three of them in sufficient numbers for the experiments. Of the other three species studied, one was shown by Stunkard (1930*a*) to be the larva of *Cryptocotyle lingua,* while the two remaining species are new to science and are described in a later section of this paper.

For these experiments it is essential that cercariæ be available in large numbers. Since cercariæ secured by crushing parasitized snails are immature and not infective (Stunkard, 1930, 1930*b*), such larvæ do not constitute suitable material, and results obtained from them are probably not significant. Consequently, only normally emerged cercariæ were used. Since several hours are required for the emergence of sufficient numbers, some of the cercariæ had been swimming for ten to twelve hours when the experiments were started. This factor undoubtedly accounts for much of the variation shown in the results. Presumably the most recently emerged cercaria lived the longest.

It is apparent in all species studied that tap water exerts an immediate and harmful effect. None of the cercariæ showed normal

Solutions	I	II	III	IV	V	VI
No. of cercariae	14	17	19	22	20	20
Time in Hours						
1	14++++	17++++	19++++	18++++ 4 encysted	12+ 8−	20−
3	13++++ 1++ (tailless)	17++++	18++++ 1++ (tailless)	14++++ 2++ 4 encysted 2−	20−	
6	12++++ 1++ 1 encysted	17++++	15++++ 3++ 1 encysted	6+ 6 encysted 10−		
15	6++++ 8 encysted	16++++ 1 encysted	15++++ 3 encysted 1+	2+ 6 encysted 14−		
22	3++ 8 encysted 3−	9++++ 8 encysted	4++ 14 encysted 1+	2+ 6 encysted 14−		
39	8 encysted 6−	11 encysted 6−	15 encysted 4−	6 encysted 18−		

activity for more than a few minutes and most of them died within an hour. The bodies and tails became swollen, the tissues underwent cytolysis with the absorption of water, and death followed shortly. Presumably there was a diffusion of salt from the organisms as water was absorbed and the loss of salt would augment and hasten the deleterious effects produced by the imbibition of water. Obviously these cercariæ can be infective for only a very brief period in tap water and it is doubtful whether they could complete their life cycle in fresh water, even if suitable hosts were available.

Larvæ placed in Solution V, containing seven-eighths tap water and one-eighth sea water, were active for considerable periods of time and some of them were able to perform swimming movements for one to four hours. Certain of them, *e.g., Cercaria parvicaudata,* appear to be deleteriously affected after a short time in this concentration, and later they partially recover. They may continue to live for several hours, although the earlier ill effects are not entirely remedied and it is doubtful whether such larvæ would be infective. They are not infective in the case of *C. lingua* and since the life histories of the other species are unknown, experimental test is impossible.

The experiments show a marked difference in the activity and longevity of cercariæ in Solutions V and IV. Whereas a solution containing one-eighth sea water is definitely harmful, cercariæ manifest little in the way of ill effects in solutions containing 25 per cent sea water. In one-fourth sea water the larvæ live almost as long as in greater concentrations, although they are usually less active after the first few hours. The sluggishness may be due to the increased water content. Cercariæ may succumb somewhat more quickly in one-fourth sea water than in more concentrated solutions, but they are normal in appearance and activity for sufficiently long periods of time to indicate that they may be infective and able to function normally in continuing the life cycle.

Considerable interest attaches to the observation that larvæ are active and apparently normal for almost if not quite as long in solutions containing 50 per cent or more of sea water as they are in undiluted sea water. In certain experiments, cercariæ actually lived longer in one-half than in undiluted sea water, although they were not normally active and probably not infective for longer periods than larvæ in sea water.

The ability of cercariæ to withstand dilution of sea water is roughly proportional to the dilution which occurs in the larger bays. Cowles (1930) reported that, "The salinity of Chesapeake Bay, like that of other long bays and estuaries, gradually decreases, with very few exceptions, from the mouth to the head; and the bay is known as a brackish body of water, although the failure as a rule, of the fresh waters from the land and the saline waters of the sea to mix completely, and the variation in the volume of fresh and salt water entering the bay, result in different degrees of brackishness. The surface data at the mouth of the bay show a variation in salinity from about 19 to 30 grams per liter, while near Baltimore there is a variation from about 3 to 11 grams per liter." . . . "The bottom salinities recorded on our cruises for the mouth of the bay varied from about 26 to a little over 32, while in the region of Baltimore they varied from about 6 to 17."

It appears more than probable that such transitional zones, extending sometimes for a distance of a hundred miles or more, with ocean water at one end and fresh water at the other, provide the ecological settings in which species become transformed physiologically and structurally from marine to fresh-water organisms, and vice versa. Due to the well-known and constantly appearing variations which occur among animals, certain fresh-water species may have become adapted to life in brackish and eventually sea water, while similarly, marine species may have entered fresh-water habitats.

If the larvæ of parasitic species are able to survive in a new and different environment long enough to find and infect a suitable host, either a former host that has migrated into the location, or a new host species, the life cycle may be completed. The long list of snails which serve as intermediate hosts of *Fasciola hepatica,* under different conditions in various parts of the world, demonstrates the extent to which that species has acquired new hosts and the work of Cort (1918) illustrates the ability of other trematode larvæ to successfully attack new hosts. In the case of a marine or brackish-water species entering fresh water, the essential factor is the ability of the free-swimming larvæ to withstand the hypotonic medium until infection is accomplished, since in the body of the host a medium of higher salt content and tonicity is encountered.

The present experiments record the ability of six marine cercariæ to withstand dilution of sea water and show that these larvæ manifest normal activity for considerable periods of time in solutions containing only one-eighth to one-fourth sea water. The observations indicate that these cercariæ are able to complete their life cycles in brackish water and denote the extent to which marine species may migrate into brackish and fresh water. The paper thus contributes toward the solution of the problem of the distribution of related species in marine and fresh-water habitats.

PLATE I

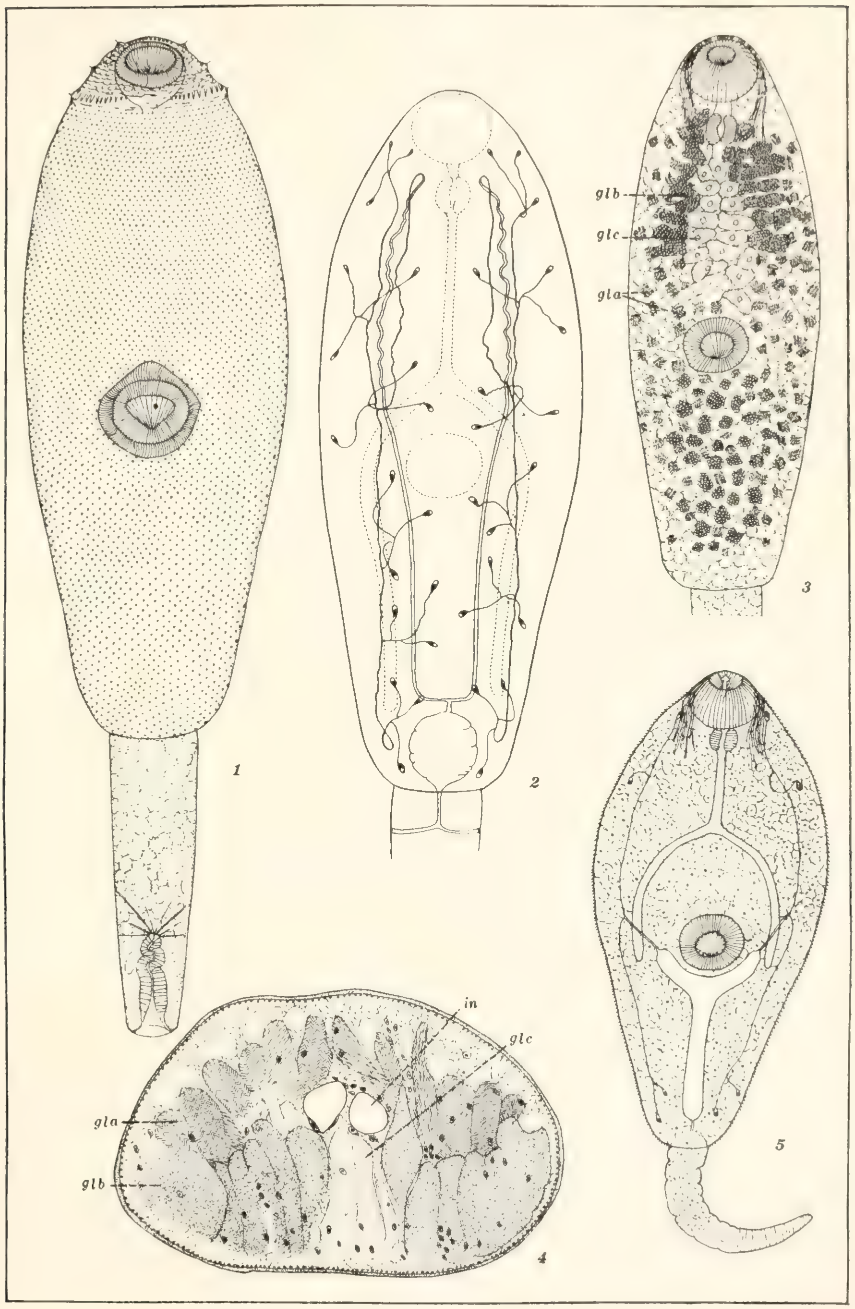

FIG. 1. *C. sensifera,* ventral view.
FIG. 2. *C. sensifera,* excretory system.
FIG. 3. *C. sensifera,* ventral view, showing distribution of gland cells.
FIG. 4. *C. sensifera,* cross section, showing three types of gland cells.
FIG. 5. *C. parvicaudata,* ventral view.

Cercaria parvicaudata n.sp.

(Fig. 5)

This species occurs in about one per cent of the specimens of *Littorina littorea* examined at Woods Hole. The cercariæ are produced in sporocysts which occupy the lymph spaces of the snail. The gonad is the principal seat of infestation and frequently this organ is entirely destroyed.

The cercariæ are small, with oval to pyriform bodies and short tails. The length of the body varies from 0.14 to 0.36 mm. in contracted and extended condition. The tail is very active; it may be contracted to a length of 0.06 mm. or extended until it exceeds the body in length. Ordinarily when the worm is attached or creeping, the tail is contracted and manifests a nervous, twitchy motion. In swimming the body is contracted into a short, wide form, bent ventrally and the tail is extended and lashes violently.

The body is covered with minute spines and the oral sucker bears a stylet 0.015 mm. long by 0.0032 mm. wide. There is a small thickening on the stylet near its tip. The acetabulum is situated near the middle of the ventral surface and measures from 0.03 to 0.05 mm. in diameter.

The cercariæ encyst readily. A portion of a dissected snail was left for six hours in a watch glass and ten cercariæ had encysted among the sporocysts. Other cysts were found in the tissue of the snail which had been fixed and later sectioned. The cysts measure approximately 0.17 mm. in diameter. There are gland cells of two types distributed throughout the parenchyma of the body; one is filled with refractive, spherical granules, the other is slightly opaque and contains very fine granules. On either side of the mouth there are openings of ducts which pass backward and appear to communicate with other glandular cells situated in the preacetabular region, although the connections of these ducts were not determined with certainty. Presumably they are the ducts of penetration or salivary glands.

The oral sucker is spherical to oval, 0.035 to 0.06 mm. in diameter. It is followed almost immediately by a small pharynx and the esophagus extends about one-half of the distance to the acetabulum. The digestive ceca terminate blindly near the level of the caudal margin of the acetabulum.

The excretory vesicle is Y-shaped, with a long stem and short branches. Its wall contains large, deeply-staining cells. Four flame cells have been definitely located and these are shown in the figure. Others were observed, but their connections were not traced. The reproductive organs are represented by a mass of cells which are dorsal and anterior to the acetabulum.

This species belongs in the large and heterogeneous group of Xiphidiocercariæ, but further attempts to relate it must await a more complete knowledge of its morphology or information concerning its adult form.

Cercaria sensifera n.sp.

(Figs. 1–4)

This species has been found only in the oyster drill, *Urosalpinx cinereus,* and it was present in fourteen out of 594 specimens examined during the summer of 1930. Six infestations were found in two hundred and six snails collected at Woods Hole during the first week of April, 1931. The parasites infest the interlobular areas of both the reproductive and digestive glands. In an uninfected snail the visceral mass is plump, the liver is yellow and the gonad is cream-colored, whereas in a parasitized snail the organs are shrunken, the gonad may be destroyed, and the body is lighter in color.

The cercariæ (Fig. 1) are large and clearly visible to the unaided eye. The body is oval in shape, more or less elongated and narrower posteriorly, flattened dorsoventrally, and widest in the preacetabular region. It is truncated posteriorly and the attachment of the tail is terminal. The cercariæ vary considerably in size and manifest much elongation and contraction in locomotion. They are not active swimmers and tend to remain near the bottom of the water. After a time swimming movements alternate with creeping ones. In swimming, the tail is elongated; it does not lash about, but the cercaria moves by undulatory movements of the body and tail. The chief propulsive force comes from the anterior half of the body. It slowly bends ventrally and then snaps backward, pulling the larva forward. The movement is continued through the posterior part of the body and tail producing the sinuous motion of the larva.

With the exception of the anterior end, the body is covered with a thick granular cuticula which bears large, closely set spines. Those in the anterior row are considerably larger than the others. This row is interrupted in the midventral region. There are 44–48 spines on the dorsal side and 10 on each side ventrally. These spines measure 0.005–0.006 mm. in length. This region of the body is sometimes contracted to produce a distinct collar-like effect. There are about forty-five annular rows of spines in the preacetabular region and about 130 to 135 such rows on the body. The spines in successive rows alternate with each other and those around the acetabulum are arranged in concentric rings. The cuticula of the tail is thin and smooth.

The larvæ are bottom forms, and attach readily to any available surface. When picked up in a pipette they frequently adhere to the inside of the tube and can be dislodged only with great difficulty. They may become attached either by the suckers or by the tip of the tail and after attachment they soon encyst. If placed in solutions that are irritating, *e.g.*, too strong concentrations of vital dyes, they encyst almost immediately. The cyst consists of two layers, a thick, opaque, external covering and a thin, transparent, very tough, inner membranous layer.

Neither of the cyst walls is readily stainable by ordinary dyes. The cyst is oval, flattened on the side of attachment, and measures from 0.2 to 0.23 mm. in width by 0.23 to 0.27 mm. in length. The tail is always detached in encystment and may remain attached for a time to the surface of the cyst. Normally the worm fills the cyst completely. The outer cyst wall is easily removed by rolling a cyst between a slide and cover glass, but it is difficult to get the worm out of the inner membranous covering without injury.

Living cercariæ may extend to a length of 0.9 mm. and contract until the length is no greater than the breadth. The tail also is capable of much extension and contraction; it may be very much shortened or elongated to almost the length of the body. In the latter condition it is slender with an expanded, cup-shaped portion at the end. The caudal tip is usually introverted in a characteristic manner (Fig. 1), although the invaginated portion may be protruded and apparently bears a sticky substance, by means of which the cercariæ adhere to objects. Fixed and stained specimens measure from 0.21 to 0.47 mm. in length and 0.14 to 0.26 mm. in width. In such specimens the tails vary from 0.12 to 0.26 mm. in length. The acetabulum is situated slightly behind the middle of the body. In living specimens it measures from 0.08 to 0.1 mm. in length and from 0.1 to 0.115 mm. in width, while in fixed and stained specimens it measures from 0.68 to 0.76 mm. in diameter.

The anterior end of the body is covered by a smooth, thin, unarmed cuticula which extends backward as far as the caudal margin of the oral sucker. This region bears a number of papillæ, arranged irregularly in two or three rows, and each papilla terminates in a bristle. Similar structures occur around the margin of the acetabulum and presumably they function in a sensory manner. This idea is expressed in the specific name, *sensifera*. The caudal one-fourth or one-fifth of the anterior unarmed area forms a zone which frequently is marked by small longitudinal furrows.

The body is filled with gland cells of several types. In the region between the pharynx and acetabulum there are twelve large unicellular glands. These cells (Figs. 3, 4, *glc*) have very small secretory granules and open to the surface through twelve pores situated at the anterior tip of the body above the oral sucker. They seem to correspond to penetration glands of other cercariæ and indicate that the larvæ at some later stage bore into the tissues of an intermediate host. The cortical layer of the parenchyma contains numerous dermal glands. In addition, the dorsal half of the body contains numerous gland cells (Figs. 3, 4, *gla*), the cytoplasm of which is filled with bacilliform granules or rods. These cells do not stain with neutral red and in sections counterstained with erythrosin the secretory products appear yellowish. The ventral portion of the body is largely filled with gland cells (Figs. 3, 4, *glb*) whose cytoplasm contains large spherical secretory granules. The cell contents stain intensely with erythrosin. In the anterior half of the body these cells appear to be arranged in four longitudinal fields, separated by the large ventrolateral nerve trunks and the esophagus.

Attempts were made to study the cercariæ in solutions of various vital dyes. With neutral red the spine-covered portion of the cuticula and the cells which secrete it quickly take up the stain and this red or pink layer at the surface of the body masks the action of the stain inside. It is clear, however, that the contents of the digestive ceca assume a deep red color. The stem of the tail, with the exception of the caudal third or fourth, is filled with large fluid globules that take the stain and become a brick-red color. Young specimens do not take the stain at all, and at a later stage the bodies of the larvæ assume a diffuse pink or rose color. The use of other stains, methylene blue, dahlia, pyronin, brilliant cresyl blue, Janus green, light green, and methyl violet did not give significant results on this cercaria. If very dilute solutions were employed the staining was slight and diffuse, not differential, and when stronger solutions were used the cercariæ encysted very quickly.

The mouth opening is subterminal and the oral sucker measures from 0.06 to 0.08 mm. in diameter in living specimens. In fixed and stained specimens the diameter is from 0.05 to 0.06 mm. There is a short prepharynx, the pharynx measures from 0.02 to 0.027 mm. in diameter, and the esophagus is long, extending about two-thirds of the distance to the acetabulum, where it bifurcates to form the intestinal ceca (Fig. 4, *in*). The ceca end blindly about two-thirds of the distance from the acetabulum to the caudal end of the body. The esophagus as well as the ceca is lined with epithelium and this point is significant for life history and taxonomic identification.

The excretory system (Fig. 2) has been worked out in detail and the pattern confirmed on dozens of cercariæ. The system forms as two separate parts, one right and the other left, as described for other cercariæ. The longitudinal ducts fuse near the posterior end of the body and this portion becomes the future excretory vesicle. With the constriction that forms the tail the posterior end of the vesicle is denoted and the two excretory pores open on either side of the tail as shown in Fig. 2. The details of the excretory system are shown in the figure. The collecting ducts extend forward to the level between the pharynx and oral sucker, passing on the ventral side of the intestinal ceca. The anterior portions of these ducts contain excretory concretions. Each collecting duct turns posteriad and this recurrent stem contains two large ciliated areas. At the level of the intestinal bifurcation the recurrent ducts divide into anterior and posterior branches. Each of the anterior and posterior branches bears three clusters of flame cells with three cells in each cluster. The cone of cilia in a flame cell measures from 0.008 to 0.01 mm. in length.

The reproductive organs are represented by a mass of deeply staining cells, the anlagen of the gonads, situated in front of the excretory vesicle, and a strand of cells which extends forward connecting with another cell mass in front of the acetabulum. The strand of cells will form the gonoducts and the cell mass in front and sometimes to the left of the acetabulum is the anlage of the genital pore and copulatory organs.

The cercariæ are produced in rediæ. A redia has two " feet," a birth pore near the oral sucker, and an intestine which contains orange-colored granules. In a small redia the germ masses are situated in the caudal third of the body and the posterior tip may be protruded in a tail-like or foot-like protuberance that is used like the feet in locomotion. There is a muscular lip-like snout in front of the oral sucker; the sucker measures from 0.05 to 0.07 mm. in diameter and in a young redia the intestine extends through three-fourths of the body length. Rediæ increase to a length of 2.1 mm. and a width of 0.4 mm. The small rediæ may have one or more fully formed cercariæ in their bodies and large rediæ contain from ten to thirty more or less developed cercariæ.

Cercaria sensifera belongs to the Megalura group of cercariæ, outlined by Cort (1915) and extended by Sewell (1922). It agrees closely with *C. purpuræ* and *C. patellæ*, marine species described by Lebour (1907, 1912) and indeed may be specifically identical with *C. purpuræ*. Slight differences between the present specimens and the account of Lebour in regard to the arrangement of the gland cells, the presence of a " neck " region, and the relations of the excretory system and the tail, make it impossible to determine with certainty whether the specimens may be referred to *C. purpuræ*. Accordingly, a new name is proposed for them with the understanding that it will disappear as a synonym if further studies prove the American species to be identical with the European. In the paper cited, Lebour (1912) predicted that the adult stage of the parasite occurs in a bird. Two years later, Lebour (1914) compared young stages of *Parorchis acanthus* Nicoll with *C. purpuræ* and identified the cercaria as the larval stage of that species, confirming a prediction made by Nicoll some years previously. Believing that *Parorchis* is closely related to the echinostomes, Lebour stated that the second intermediate host is probably a mollusk. In a later report, Lebour and Elmhirst (1922) reported that *C. purpuræ* encysted in the mantle of *Cardium edule* and *Mytilus edulis*. Their figure of the cyst from side view indicates that it is on the surface rather than embedded in the mantle of the host. The observations of Lebour and Elmhirst appear to complete the life cycle of the species but the experiments were not sufficiently controlled to exclude other possibilities. In the first and only successful experiment reported, the cercariæ used " swam actively by a strong side to side lashing of the tail." It is questionable whether these larvæ were actually *C. purpuræ* since megalurous cercariæ do not employ this method of swimming. Two types of larval trematodes were found encysted in the single specimen of *Cardium* used in the experiment and the more abundant species was identified as *Echinostomum secundum*. Consequently, two types of cercariæ were introduced unless the intermediate host was already infected when the experiment was begun. Apparently the authors did not know whether or not the bivalves used in the experiment were previously infected, and results of such experiments are not at all conclusive. If *C. purpuræ,* like *C. sensifera* will encyst on any surface to which it adheres, it is only natural that the larvæ should encyst on the mantle of mollusks placed in aquaria with them, and it does not necessarily follow that these mollusks are the normal intermediate hosts.

The idea at once presents itself that *C. sensifera* is a larval stage of *Parorchis avitus* Linton, 1914, an unusual trematode in which free miracidia were found in the uterus and in which each miracidium contained a well developed redia. Such a life cycle would explain the observation of Lebour that sporocysts or other preredial stages of *C. purpuræ* were not found in the snail host. Linton (1928) gave a further discussion of *P. avitus* and argued against the suggestion of Nicoll that the American species is identical with *Parorchis acanthus*.

SUMMARY

The problem concerning the origin and distribution of closely related parasites that occur in marine and fresh-water hosts is discussed. Migration of hosts and transfer to new hosts are the only explanations that appear tenable. The essential factor is the ability of the free-swimming larvæ to live and remain infective in a changed environment. Experiments on the ability of six species of marine cercariæ to withstand dilu-

tion of sea water show that these larvæ manifest normal activity for considerable periods of time in solutions containing only one-eighth to one-fourth sea water. The observations indicate that these cercariæ are able to complete their life cycles in brackish water and denote the extent to which these organisms may migrate into brackish or fresh water.

BIBLIOGRAPHY

Adolph, E. F., 1925. Some Physiological Distinctions Between Freshwater and Marine Organisms. *Biol. Bull.*, **48**: 327.

Allee, W. C., 1923. Studies in Marine Ecology. III. Some Physical Factors Related to the Distribution of Littoral Invertebrates. *Biol. Bull.*, **44**: 205.

Bresslau, E., and E. Reisinger, 1928. Allgemeine Einleitung zur Naturgeschichte der Platyhelminthes. In Handbuch der Zoologie, Kükenthal-Krumbach, Berlin and Leipzig.

Cort, W. W., 1915. Some North American Larval Trematodes. *Ill. Biol. Monogr.*, **1**: 447.

Cort, W. W., 1918. Adaptability of Schistosome Larvæ to New Hosts. *Jour. Parasit.*, **4**: 171.

Cowles, R. P., 1930. A Biological Study of the Offshore Waters of Chesapeake Bay. *Bull. Bur. Fish.*, **46**: 277.

Fuhrmann, O., 1928. Trematoda. In Handbuch der Zoologie, Kükenthal-Krumbach, Berlin and Leipzig.

Hay, O. P., 1908. The Fossil Turtles of North America. *Carnegie Inst. Wash.*, Pub. No. 75.

Lebour, M., 1907. Larval Trematodes of the Northumberland Coast. *Trans. Nat. Hist. Soc. Newcastle*, N.S., **1**: 437–454, 500–501.

Lebour, M. V., 1912. A Review of the British Marine Cercariæ. *Parasit.*, **4**: 416.

Lebour, M. V., 1914. Some Larval Trematodes from Millport. *Parasit.*, **7**: 1.

Lebour, M., and R. Elmhirst, 1922. A Contribution Towards the Life-History of Parorchis acanthus Nicoll, a Trematode in the Herring Gull. *Jour. Mar. Biol. Ass. Plymouth*, N.S., **12**: 829.

Linton, E., 1928. Notes on Trematode Parasites of Birds. *Proc. U. S. Nat. Mus.*, **73**: 1.

Looss, A., 1902. Ueber neue und bekannte Trematoden aus Seeschildkröten. *Zool. Jahrb., Syst.*, **16**: 411.

Mackin, J. G., 1930. A New Pronocephalid Monostome from a Freshwater Turtle. *Jour. Parasit.*, **17**: 25.

Manter, H. W., 1926. Some North American Fish Trematodes. *Ill. Biol. Monogr.*, **10**: No. 2, p. 7.

Marshall, E. K., Jr., and H. W. Smith, 1930. The Glomerular Development of the Vertebrate Kidney in Relation to Habitat. *Biol. Bull.*, **59**: 135.

Meixner, J., 1926. Beitrag zur Morphologie und zum System der Turbellaria-Rhabdocoela. II. *Zeitschr. wiss. Biol.*, Abt. A., Morph. u. Okol., **5**: 577.

Miller, H. M., and F. E. Northup, 1926. The Seasonal Infestation .of Nassa obsoleta (Say) with Larval Trematodes. *Biol. Bull.*, **50**: 490.

Needham, J., 1930. On the Penetration of Marine Organisms into Freshwater. *Biol. Zentralbl.*, **50**: 504.

Nicoll, W., 1915. A List of the Trematode Parasites of British Marine Fishes. *Parasit.*, **7**: 339.

Nicoll, W., 1924. A Reference List of the Trematode Parasites of British Freshwater Fishes. *Parasit.*, **16**: 127.

Pantin, C. F. A., 1931. The Adaptation of Gunda ulvæ to Salinity, III. *Jour. Exper Biol.*, **8**: 82.

REISINGER, E., 1928. Allgemeine Einleitung zur Naturgeschichte der Vermes Amera. In Handbuch der Zoologie, Kükenthal-Krumbach, Berlin and Leipzig.

SEWELL, R. B. S., 1922. Cercariæ indicæ. *Ind. Jour. Med. Res.*, **10**: Suppl. No.: 1.

STUNKARD, H. W., 1917. Studies on North American Polystomidæ, Aspidogastridæ, and Paramphistomidæ. *Ill. Biol. Monogr.*, **3**: 285.

STUNKARD, H. W., 1929. The Parasitic Worms Collected by the American Museum of Natural History Expedition to the Belgian Congo, 1909–1914. *Bull. Am. Mus. Nat. Hist.*, **58**: 233.

STUNKARD, H. W., 1930. Morphology and Relationships of the Trematode Opisthoporus aspidonectes (MacCallum, 1917), Fukui, 1929. *Trans. Amer. Micr. Soc.*, **49**: 210.

STUNKARD, H. W., 1930*a*. The Life History of Cryptocotyle lingua (Creplin), with Notes on the Physiology of the Metacercariæ. *Jour. Morph. and Physiol.*, **50**: 143.

STUNKARD, H. W., 1930*b*. An Analysis of the Methods used in the Study of Larval Trematodes. *Parasit.*, **22**: 268.

STUNKARD, H. W., 1930*c*. The Effect of Dilution of Sea-Water on the Activity and Longevity of the Cercariæ of Cryptocotyle lingua. *Anat. Rec.*, **47**: 362.

STUNKARD, H. W., AND C. H. ALVEY, 1930. The Morphology of Zalophotrema hepaticum, with a Review of the Trematode Family Fasciolidæ. *Parasit.*, **22**: 326.

STUNKARD, H. W., AND R. F. NIGRELLI, 1930. On Distomum vibex Linton, with Special Reference to its Systematic Position. *Biol. Bull.*, **58**: 336.

TENNENT, D. H., 1906. A Study of the Life History of Bucephalus haimeanus; a Parasite of the Oyster. *Quart. Jour. Micr. Sci.*, **49**: 635.

WILLISTON, S. W., 1914. Water Reptiles of the Past and Present. Univ. of Chicago Press, Chicago, Ill.

WOODHEAD, A. E., 1929. Life History Studies on the Trematode Family Bucephalidæ. *Trans. Amer. Micr. Soc.*, **48**: 256.

WOODHEAD, A. E., 1930. Life History Studies on the Trematode Family Bucephalidæ. No. II. *Trans. Amer. Micr. Soc.*, **49**: 1.

ZITTEL, K. A., AND C. R. EASTMAN, 1913. Textbook of Paleontology, 2d ed., Vol. 1.

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THE TENSION AT THE SURFACE OF MARINE EGGS, ESPECIALLY THOSE OF THE SEA URCHIN, ARBACIA

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Biologists frequently speak of the surface tension of cells, comparing their form, movements and division to phenomena connected with surface tension at oil-water interfaces. It seems unlikely that the tension is a true surface tension between non-misceable fluids, but the behavior of deformed spherical cells shows very clearly that a surface force exists which can best be referred to as the *" tension at the surface "* without implying either elastic tension or surface tension. Estimates of its magnitude have been made in different ways and until recently the values obtained have been relatively high, from 10 to 50 dynes per centimeter. I believe the tension is very much smaller than this in many cells.

In a recent paper (1931) I have described an approximation method for determining the tension at the surface of an unfertilized egg in sea water, from the centrifugal force necessary to pull the egg apart. In the worm, *Chætopterus pergamentaceus,* the forces necessary are small and the whole process can be observed and photographed in the microscope-centrifuge (Harvey and Loomis, 1930), whose maximum speed is 4000 R.P.M. The value obtained for *Chætopterus* was about one dyne per centimeter, which represents the *maximum* value, since all the assumptions made were such as to give a maximum. The true value is probably considerably less than this, but the fact that the surface forces are so low is a point of great interest.

The method does not allow us to decide whether this force is a true surface tension at a liquid-liquid interface or the elastic tension of a membrane, because we cannot tell whether or not the strain is independent of the stress. Micro-dissection studies (Chambers, 1921) indicate that the surface of marine eggs is surrounded by an actual consistent film variously spoken of as the " pellicle " or vitelline membrane, which lifts off and hardens to form the fertilization membrane of *Arbacia* eggs. Since a *thin* elastic film will take the same configuration under distortion as a surface showing true surface tension, and since the pellicle in the egg becomes discontinuous at the time of cell division or when the egg is fragmented by centrifuging, we may regard such a film as having the properties of a true surface (except for the relation between stress and strain), and on this basis calculate its tension by methods which at least give order of magnitude and a maximum value.

The argument for the *Arbacia* egg is somewhat different from that used in the case of *Chætopterus,* because *Arbacia* pulls apart in a quite different manner. In *Chætopterus* in sea water an oil spherule pulls away from the rest of the egg at 4000 R.P.M. (11 cm. radius), remaining attached to the egg by a long stalk pulled out to many times the diameter. The picture is the same if the eggs are suspended in a sugar solution of the same density or of greater density, when the eggs float. In the latter case a yolk spherule is pulled away from the remainder of the egg, again with a long connecting stalk.

Unfertilized eggs of the sea urchin, *Arbacia punctulata,* cannot be rapidly pulled apart with this force but, at about 7000 R.P.M. (same radius), if suspended in a medium of the same density as the egg, they elongate, form dumb-bell shapes and in 4 minutes separate into a lighter and a heavier half of nearly the same size. The egg may be regarded as a sphere pulled into a cylinder with rounded ends by the buoyant force of the oil and the weight of the heavier yolk mass. In this process the surface area increases about 25 per cent.

It is well known that a cylinder of fluid becomes an unstable form when its length exceeds its circumference ($2\pi r$), *i.e.,* when its length is about three times its diameter (Lord Rayleigh, 1879). Under these circumstances it will divide into two. The surface tension (σ) around the circumference should then just balance the forces pulling the cylinder apart, $\sigma 2\pi r =$ forces stretching cylinder.

If we regard the egg as a sphere non-misceable with sea water, we can calculate its circumference when drawn into a cylinder with hemispherical ends whose length is π times its diameter. The breaking up into two spheres of such a form will only be delayed because of the viscosity of the sphere.

We wish to know the radius of a cylinder of height, h, with hemispheres at each end of radius, r, in which $h + 2r = 2\pi r$ and whose volume equals that of a sphere, the *Arbacia* egg, of diameter, d.

Hence, $\pi r^2 h + 4/3\pi r^3 = 4/3\pi(d/2)^3$

But, $h = \pi 2r - 2r$

Substituting, $\pi r^2(\pi 2r - 2r) + 4/3\pi r^3 = 4/3\pi(d/2)^3$

or $2\pi^2 r^3 - 2\pi r^3 + 4/3\pi r^3 = 4/3\pi(d/2)^3$.

Since the average diameter of an *Arbacia egg* is 74 μ,

$$17.65r^3 = 212000\ \mu^3$$
$$r = 23.0\ \mu.$$

The forces pulling the egg apart are due to the weight of the heavy fragment (H) and the buoyancy of the light fragment (L), which can be determined from the volume of the fragments (V), their densities (ρ), and the density of the medium (ρ_M) which is equal to the density of the whole egg since the eggs are centrifuged in a medium of equal density.

Force (in dynes) $= V_H\ (\rho_H - \rho_M) + V_L\ (\rho_M - \rho_L) \times 980 \times C$, where C = centrifugal force in terms of gravity.

Since the egg pulls into approximately equal parts, the density of the light fragment must be as much less than the medium as the density of the heavy fragment is greater than the medium.[1] If V_E is the volume of the egg, the whole relation therefore becomes:

$$2\pi r\sigma = V_E\ (\rho_M - \rho_L) 980 \times C. \qquad (1)$$

It is only necessary to know the diameter of an egg, the density of the medium and that of the light fragment and the centrifugal force to divide the eggs.

The diameter of the *Arbacia* egg (74μ) gives a volume (V_E) of 2.12×10^{-7} cm.^{3} To get the density of the eggs (without jelly) they must be suspended in a medium of the same osmotic pressure as well as the same density. Lucké has found that cane sugar of .95 molal concentration (342 grams cane sugar added to 1 liter water) causes neither swelling nor shrinking of *Arbacia* eggs.[2] I find that when centrifuged in one part of sea water and 3 parts of .95 M cane sugar, some

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[1] Dr. Balduin Lucké, in the course of some experiments on the osmotic properties of yolk and clear halves, has measured the volumes in cubic micra and compared the sum of these volumes with the volume of the original egg. Each figure is the mean of 50 cells and must be multiplied by 100.

Date	Control egg *A*	Colorless half *B*	Yolk half *C*	Sum of *B* and *C*
Aug. 7........	1895	1013	939	1952
" 8........	2088	1185	932	2117
" 13........	1954	1129	814	1943
" 14........	1905	983	904	1887
" 15........	2030	1120	945	2065

The yolk half is somewhat smaller than the colorless half, averaging around 11 per cent smaller.

[2] Private communication. See also Lucké, 1931. *Biol. Bull.*, **60**: 75.

lots of eggs float, most sink and some remain suspended even under high centrifugal forces. This mixture has a density of 1.081 at 23° C.[3] In one part sea water and four parts .95 M cane sugar the eggs of most females float; others sink very slowly. Its density is 1.085 at 23° C. We may consider the density of the medium (ρ_M) and the egg to be 1.083. I believe Heilbrunn's (1926) value of 1.0485 (12.5 per cent sugar) and 1.0656 (16.5 per cent sugar) for different lots of eggs are too low, because he suspended the eggs in pure sugar solutions which were hypotonic and volume changes must have occurred. However, the density of different lots of eggs does vary considerably. Eggs with jelly have a density of about 1.090, whereas without jelly the density is about 1.083 to 1.084.

To obtain the density of the light fragments (ρ_L), the eggs (*without jelly*) are centrifuged in the sugar-sea water mixture of the same density and the light fragments transferred to other mixtures of sugar and sea water. They mostly float in 10 parts of sea water to 20 parts .95 M sugar, whose density is 1.076, whereas they all sink in 12 parts sea water to 20 parts .95 M sugar, whose density is 1.073. We may therefore consider their density to be 1.075 and $\rho_M - \rho_L = .008$.

The sugar-sea water mixture of the same density as the egg is not toxic. Although eggs will not fertilize in the sugar solution, they can be fertilized and develop normally when removed to sea water after an immersion of five hours.

In determining the centrifugal force the time factor is an important consideration. Part of the time is involved in the separation of granules of different density within the eggs. Only when this happens do the stretching forces appear. Part of the time is connected with the slow pinching of the egg in two. Even when an egg has assumed a prolate spheroid shape, recovery of the spherical form is very slow, a matter of many minutes. Dumb-bell-shaped eggs do not pinch in two after the centrifugal force is removed but remain dumb-bells for many minutes, gradually becoming spherical again (after 40 minutes). There is also considerable variation in the ease with which eggs can be pulled apart. Eggs from some females fragment at 122 r.p.s. in 4 minutes, others do not but will fragment in 12 minutes. Eggs which fragment in 4 minutes at 122 r.p.s. are not pulled apart in 4 minutes at 112 r.p.s., but fragment in 12 minutes. The same eggs centrifuged for 20 minutes at 100 r.p.s. do not fragment but do in 24 minutes. The eggs of some females pulled apart at 60 r.p.s. in 30 minutes, but not at 50 r.p.s. in 90 minutes.

[3] Densities were determined with a hydrometer calibrated for 15° C./15° C., reading to the third decimal place. The temperature correction will be small.

EDITOR'S NOTE:

The equation on page 277 of the article by E. N. Harvey in the December, 1931, issue should be corrected to read as follows:

$$\sigma = \frac{2.12 \times 10^{-7} \times .8 \times 10^{-2} \times 10^{3} \times 1.6 \times 10^{3}}{6.28 \times 23 \times 10^{-4}} = 0.19 \text{ dynes per cm.}$$

$$\sigma = \frac{2.12 \times 10^{-7} \times .8 \times 10^{-2} \times 10^{3} \times 1.6 \times 10^{3}}{6.28 \times 23 \times 10^{-4}} = 0.19 \text{ dynes per cm.}$$

The result is very much lower than the 10 to 25 dynes per centimeter observed by Vlès (1926) for the egg of another sea urchin, probably *Paracentrotus.*

Should the eggs rest on a surface so that only the buoyant force of the light half is operative, the value will be about one-half of the above. The stretching forces must act against not only a tension at the surface, but viscous forces of the egg as well, which again will lower the figure. Finally, the calculation is based on the view that the tension is a true surface tension. If the elastic tension of a pellicle is involved, we are observing its breaking strength and its tension must be considerably less for a given stretch. It must be emphasized that 0.2 dyne per centimeter is not a very accurate value, but a maximum one, and again illustrates the very low tension at the surface of eggs presumably surrounded with a pellicle. The question arises as how general this order of magnitude is for other marine eggs.

Dr. H. K. Hartline has pointed out to me that fluid spheres from which a small oil spherule is drawn out should become unstable when the neck connecting oil spherule with the sphere has the same diameter as the spherule. The spherule will then pinch off as a separate drop. In this case the surface tension around the circumference of the oil spherule should counterbalance the buoyant force of the oil. In the case of *Chætopterus* eggs, the separation of the oil spherule occurs only after a long stalk has been pulled out and the buoyant force of the oil was regarded as counterbalancing the tension around the circumference of the stalk, 9 μ in diameter (Harvey, 1931). If the circumference of the oil spherule is considered, 34 μ in diameter, the value for the tension at the egg surface comes out about one-quarter of 1.32 dynes, or .33 dynes per centimeter.

about 1.083 to 1.084.

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In determining the centrifugal force the time factor is an important consideration. Part of the time is involved in the separation of granules of different density within the eggs. Only when this happens do the stretching forces appear. Part of the time is connected with the slow pinching of the egg in two. Even when an egg has assumed a prolate spheroid shape, recovery of the spherical form is very slow, a matter of many minutes. Dumb-bell-shaped eggs do not pinch in two after the centrifugal force is removed but remain dumb-bells for many minutes, gradually becoming spherical again (after 40 minutes). There is also considerable variation in the ease with which eggs can be pulled apart. Eggs from some females fragment at 122 r.p.s. in 4 minutes, others do not but will fragment in 12 minutes. Eggs which fragment in 4 minutes at 122 r.p.s. are not pulled apart in 4 minutes at 112 r.p.s., but fragment in 12 minutes. The same eggs centrifuged for 20 minutes at 100 r.p.s. do not fragment but do in 24 minutes. The eggs of some females pulled apart at 60 r.p.s. in 30 minutes, but not at 50 r.p.s. in 90 minutes.

[3] Densities were determined with a hydrometer calibrated for 15° C./15° C., reading to the third decimal place. The temperature correction will be small.

If eggs which have been pulled into dumb-bell form at 120 r.p.s. are observed in the microscope centrifuge at 60 r.p.s., a few pull apart into two spheres. The connecting strand between the spheres does not become long and fine as in the pulling off of an oil spherule in *Chætopterus,* but the break can be observed to occur when the length is about three times the diameter of the resultant half egg.

Since the centrifugal force (C) in terms of gravity is given by $C = .443n^2$, for 11 cm. radius, where $n =$ revolutions per second, C is 6380 for 120, 5560 for 112, 4430 for 100, and 1595 for 60 r.p.s.

Selecting a speed of 60 r.p.s., and inserting in (1), we have:

$$\sigma = \frac{2.12 \times 10^{-7} \times .8 \times 10^{-2} \times 10^{3} \times 1.6 \times 10^{3}}{6.28 \times 23 \times 10^{-4}} \quad 0.19 \text{ dynes per cm.}$$

The result is very much lower than the 10 to 25 dynes per centimeter observed by Vlès (1926) for the egg of another sea urchin, probably *Paracentrotus.*

Should the eggs rest on a surface so that only the buoyant force of the light half is operative, the value will be about one-half of the above. The stretching forces must act against not only a tension at the surface, but viscous forces of the egg as well, which again will lower the figure. Finally, the calculation is based on the view that the tension is a true surface tension. If the elastic tension of a pellicle is involved, we are observing its breaking strength and its tension must be considerably less for a given stretch. It must be emphasized that 0.2 dyne per centimeter is not a very accurate value, but a maximum one, and again illustrates the very low tension at the surface of eggs presumably surrounded with a pellicle. The question arises as how general this order of magnitude is for other marine eggs.

Dr. H. K. Hartline has pointed out to me that fluid spheres from which a small oil spherule is drawn out should become unstable when the neck connecting oil spherule with the sphere has the same diameter as the spherule. The spherule will then pinch off as a separate drop. In this case the surface tension around the circumference of the oil spherule should counterbalance the buoyant force of the oil. In the case of *Chætopterus* eggs, the separation of the oil spherule occurs only after a long stalk has been pulled out and the buoyant force of the oil was regarded as counterbalancing the tension around the circumference of the stalk, 9 μ in diameter (Harvey, 1931). If the circumference of the oil spherule is considered, 34 μ in diameter, the value for the tension at the egg surface comes out about one-quarter of 1.32 dynes, or .33 dynes per centimeter.

Similar reasoning applied to the fertilized egg of the mollusk, *Illyanassa obsoleta,* in which the oil can be observed to pull off as with *Chætopterus* in the microscope centrifuge, gives a value of 1.1 dynes per centimeter.

The eggs of the mollusk, *Cumingia tellinoides,* in sea water behave differently. They pull out into long cylinders 5 to 8 times their width, when the oil separates as a spherule which floats to the surface. Other clear spherules may separate also. The oil spherules are about 25 μ in diameter while the original egg is some 62 μ in diameter. If we assume that unstable conditions appear when the egg is pulled out to a cylinder whose diameter equals that of the oil spherule, we can calculate roughly the tension at the surface as follows:

$$\sigma \pi d = V_o(\rho_w - \rho_o) \times g \times C,$$

where σ = tension at surface, d = diameter of oil spherule, ρ_w = density sea water, 1.025, ρ_o = density of oil, C = centrifugal force in terms of gravity, g, and V_o = volume of the oil. Assuming the density of the oil to be .925 and observing that these eggs pull apart in 8 minutes at 122 r.p.s. (11 cm. radius), we have

$$78.5 \times 10^{-4}\sigma = 6.42 \times 10^{-9}(1.025 - .925) \times 10^{3} \times 6.6 \times 10^{3}$$
$$\sigma = .54 \text{ dynes per centimeter.}$$

Again the tension at the surface comes out a low value.

The unfertilized egg of the worm, *Nereis,* possesses a definite membrane. Its granules cannot be stratified by easily attainable centrifugal forces. However, the fertilized egg of *Nereis* can be stratified but cannot be fragmented, even at 17000 times gravity, although a slight tendency to elongate occurs. The membrane of *Nereis* is very strong. Calculations from the amount of oil (assuming its density = .925) present in the *Nereis* egg indicate that the tension to withstand 17000 times gravity must be considerably greater than 24 times that of the egg of *Chætopterus.*

SUMMARY

Calculations from the centrifugal force necessary to pull an *Arbacia* egg into two nearly equal parts, a yolk half and a clear half, indicate that the tension at the surface for 25 per cent increase in area is less than 0.2 dyne per cm., with considerable variation in different eggs.

Similar calculations based on the force necessary to pull an oil spherule away from the remainder of the egg give maximum values of 0.33 dynes per centimeter for *Chaetopterus,* 1.1 dynes per centimeter for *Illynassa* and 0.54 dyne per centimeter for *Cumingia.*

LITERATURE CITED

Chambers, R., 1921. *Biol. Bull.,* **41**: 318.
Harvey, E. N., 1931. *Biol. Bull.,* **60**: 67.
Harvey, E. N., and A. L. Loomis, 1930. *Science,* **72**: 42.
Heilbrunn, L. V., 1926. *Jour. Exper. Zool.,* **44**: 255.
Lord Rayleigh, 1879. *Proc. Roy. Soc.,* **29**: 83.
Vlès, F., 1926. *Arch. d. Physique Biol.,* **4**: 263.

THE THEORY OF MITOGENETIC RADIATION

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Introduction

It has long been supposed that nuclear and cell division may be affected by external as well as internal factors. While it is true that increase in temperature will accelerate division rate and many means are known to retard or prevent cell division, the evidence that mitosis, except in the case of special tissues, can be initiated or accelerated by definite compounds or by any means (except heat) is far from convincing. This is particularly true in the case of mitogenetic rays of Gurwitsch (1923), rays given off by cells or cell extracts that will induce division in another cell.

This theory, which has many opponents as well as advocates, is set forth at some length in Gurwitsch's monograph (1926) and an article in Protoplasma (1929), recently reviewed by Hollaender and Schoeffel (1931). It is based upon the following observations: if an onion root is placed vertically so that the meristematic area is perpendicular to a second, horizontally placed, root, at a distance of not more than 4 cm., and left in this position for at least 20 minutes, then, when sections are made of the first root, it can be seen on counting the number of dividing cells in the two halves of this root that cell division was markedly stimulated in the area exposed to the second root. The induced increase in cell division in such an experiment may be as great as 80 per cent. The area affected is always small, extending rarely for more than 50 micra. This mitogenetic effect is equally as pronounced whether the roots are in air or in water during the induction; the effect is not markedly lessened by the interposition of a sheet of quartz between the " sender " and " detector " roots, but is completely obliterated if a sheet of glass is placed between the two roots. The further observation that this mitogenetic influence, besides being propagated in a rectilinear manner, was capable of regular rectilinear reflection led Gurwitsch and Frank (1927) to conclude that this influence, or M-ray, was in nature identical with ultra-violet light. By comparing the induction effect of one root on another with the mitogenetic effects of ultra-violet light of varying wave lengths on a similar root, a wave length of 1900–2300 angstroms has been ascribed to the M-ray. This conclusion was influenced to some extent by the observations of Rawin (1924), who has shown that, while the M-rays are effective in inducing cell division for a distance of very nearly 4 cm., they can only penetrate glass for a distance of 50 micra before being completely absorbed.

Reiter and Gabor (1928), in a long series of similar studies on the reflection, refraction, diffraction, and absorption of the mitogenetic rays, find that they show the same properties in respect to these phenomena as ultra-violet light with a wave length of 3300 to 3400 angstroms, with a second maximum at 2800 A°.

Frank (1929) ascribes this difference in the assigned wave length of the M-rays to the two sets of workers having used different intensities of ultra-violet light in making their comparative studies. In spite of their difference of opinion over the wave length of the M-rays, they all agree that they are identical with ultra-violet light.

The experiments of Gurwitsch received almost immediate confirmation by a group of Russian workers and somewhat later by a smaller group of non-Russian workers. These have extended the known sources of mitogenetic rays until it includes the following tissues: *root tips and other embryonic plant structures,* Gurwitsch (1923), Gurwitsch, A., and N. (1924), Rawin (1924), Frank and Salkind (1926), Wagner (1927), Baron (1926), Reiter and Gabor (1928), Borodin (1930), Stempell (1929), Hollaender and Schoeffel (1931); *potato leptom,* Kisliak Stratkewitsch (1927); twenty-four-hour-old *sterile beet pulp,* Anna Gurwitsch, as quoted by Gurwitsch (1929); *tadpole heads,* Gurwitsch, A., and L. (1925), Rusinoff (1925), Reiter and Gabor (1928); *brain* of young tadpole, Anikin (1926); *bacteria,* J. and M. Magrou (1927, 1928), J. and M. Magrou and Choucroun (1929), Baron (1926, 1928), Borodin (1930); *yeast,* Baron (1926), Gurwitsch (1926, 1929), Reiter and Gabor (1928), Borodin (1930), Hollaender and Schoeffel (1931); *sea urchin eggs,* Frank and Salkind (1927), Salkind (1929), Frank and Kurepina (1930); *animal half of amphibian* morula, Anikin (1926); *yolk of chicken egg,* Sorin (1928); *corneal epithelium of starved rats, triton, and frog,* Gurwitsch, L., and Anikin (1928); *contracting muscle,* Siebert (1928), Frank and Popoff (1929); *isolated frog heart,* Salkind, Potozky, and Zoglina (1930); *Jenson sarcoma,* Siebert (1928); *malignant tumors,* Reiter and Gabor (1928), Hollaender and Schoeffel (1931); *bone marrow, spleen and lymph glands* of young rats, Sussmonowitsch, quoted from Gurwitsch (1929); confirmed for *bone marrow* by Siebert (1928); *active isolated nerves,* Wassiliew, Frank, and Goldenberg (1931); *reabsorption processes* accompanying amphibian metamorphosis, Blacher (1930), Blacher and Bromley (1930), Blacher and Holzmann (1930), Bromley (1930) and Holzmann (1930); both *normal and hemolysed blood* of frog and rat, Gurwitsch, A. and L. (1926), and Sorin (1926); *blood and urine* of healthy persons, Siebert, W. W. (1930), Gurwitsch, A. and L. (1928), Hollaender and Schoeffel (1931), Gesenius, H. (1930), Potozky and Zoglina (1928).

The M-rays are thought to take their origin in some oxidative reaction connected with normal metabolic processes of the tissue emitting the ray and not necessarily to cell division. Frank and Popoff (1929) attribute the rays emanating from muscular contraction to the explosive decomposition of glycogen to lactic acid, while Siebert (1928) thinks they originate in the oxidation of lactic acid. The latter to prove his point has constructed several chemical models, oxidative reactions in test-tubes, which imitate the radiating properties of living tissues.

Gurwitsch (1924, 1925), however, attributes the origin of the rays to an enzymatic reaction similar to the oxidation of luciferin by luciferase. In the latter reaction visible light is emitted, while in the former there is an emission of ultra-violet-like M-rays. To prove his point, Gurwitsch has succeeded in extracting from a pulp of onion roots, by following the procedure of Dubois, two substances which are separately inactive, but which on being mixed will emit M-rays for as long as an hour. He calls one of these substances " mitotin," and the other, which he thinks an enzyme, " mitotase."

So far the proof of the existence of a mitogenetic radiation has been entirely physiological and rests upon the ability of the rays to induce an increased cell division in the meristem of onion roots, in yeast and bacterial cultures, eggs and other tissues with by far the greater emphasis being placed on the onion root as a detector of the radiation.

The Gurwitsch school claims that normally there is a radial symmetry in the distribution of mitoses in an onion root, and that there is never in a normal root a deviation of more than 10 per cent from this symmetry.

The chief proof of the existence of the mitogenetic radiation is its ability to destroy this symmetry by increasing by 20 to 80 per cent the number of dividing cells in the area exposed to the radiation.

A good many of the workers on these rays have been content to accept the assertion of the Gurwitsch school as to the existence of this symmetry (*e.g.*, Borodin, 1930) and rather than undertake the laborious and extremely tedious task of counting the dividing cells in normal roots in addition to making the necessary counts for their experimental roots, have been publishing the results of experiments that were very inadequately controlled. With these workers any variation from the " normal " symmetry that was greater than 10 per cent was all too apt to be regarded as being evidence of a mitogenetic influence.

Obviously as soon as any doubts are cast upon the actuality or universality of this radial symmetry the mass of evidence based entirely on the existence of such a symmetry becomes questionable. Such is the case at present, for in the recent work of Schwarz (1928), Rossmann (1928), and von Guttenberg (1928, *a* and *b*), the existence of such a symmetry as claimed by the Gurwitsch school is emphatically denied.

Schwarz in eight experiments in which he used onion roots as sources as well as detectors of the M-rays obtained positive results of 15.6 per cent, 12 per cent, and 0.8 per cent respectively in 3, and negative effects of 2.2 per cent, 5.4 per cent, 5.5 per cent, 17.7 per cent, and 22.7 per cent in the other five experiments.

Rossmann and von Guttenberg in studying normal unexposed roots found a variation from symmetry as high as 32 per cent in onion roots, and 38 per cent in pea roots. In a large number of experiments they were unable to obtain a mitogenetic effect greater than this variation which they found in their normal untreated roots.

Gurwitsch (1928, *a* and *b*), in his reply to the criticism of the above workers, reaffirms his conclusion that normally there is no more than 10 per cent variation. He criticises the technique of Rossmann in his focussing of the sender root on the detector root, and in his handling of the roots after treatment.

It is of significance that Wagner (1927) was only able to obtain a mitogenetic effect from exposing one onion root to another when the detector root contained relatively few dividing cells. With these small numbers even a slight difference when expressed as a percentage difference would seem large.

Additional negative evidence has been offered by Choucroun (1930), Urbanowicz (1927), and Rossmann (1928–29). Choucroun was unable to duplicate in later experiments the results obtained by J. and M. Magrou and himself in the work referred to above (1929). He concludes that the abnormalities appearing in sea urchin eggs on exposure to a bacterial culture, and formerly attributed to a mitogenetic radiation from the bacteria, were due to an actual passage of some substance from the culture to the dishes containing the eggs. No effect could be obtained when the vessels containing the eggs were tightly sealed. Urbanowicz could not increase the rate of division in *Paramecium* by exposing them to onion roots. Rossmann, in answer to Gurwitsch's criticism of his earlier work, performed 47 experiments in which yeast and onion roots were used both as senders and detectors of the M-rays. In only one of the 47 experiments did he obtain a positive effect.

The lack of agreement between the Gurwitsch school on the one hand and Schwarz, Rossmann, and von Guttenberg on the other as to

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the symmetry of the onion root, and the rather inconclusive experiments of Wagner led us to study the distribution of dividing cells in the meristem of several onion roots which had not been exposed to any supposed source of a mitogenetic radiation.

Experimental

The roots, grown in the laboratory to a length of 2–10 millimeters, were fixed in Bouin's as modified by McClung. Cross-sections were made 7–12 micra in thickness. They were stained with iron hematoxylin. Only those series that had been cut symmetrically were used. This was to insure that any asymmetry observed could not be attributed to an asymmetrically cut section.

In making our counts the number of cells in each quadrant of the section was counted separately, then by adding the numbers obtained in two adjacent quadrants one-half of each section could be compared with the other, thus making it possible to compare the distribution of mitoses on the two sides of each of two diameters for each section of the root. A cell was considered to be in mitosis from the earliest recognizable spireme stage until the two daughter cells had been completely separated. In the following tables the halves 1 + 2 are compared with 3 + 4, and 1 + 4 with 2 + 3, the location of the quadrants on the section being indicated in the small circle at the top of the table by the numbers 1, 2, 3, and 4.

Table I shows the distribution of dividing cells as noted in one root, counted for 33 sections in the center of the meristematic area. When the halves 1 + 2 and 3 + 4 are compared there is a maximal variation of 31 per cent in one direction and 27 per cent in the other. Nineteen out of the 33 sections counted showed a variation of more than 10 per cent. When the halves 1 + 4 and 2 + 3 are compared 14 of the 33 sections show an asymmetry of more than 10 per cent. The maximal variation is 14 per cent in one direction and 31 per cent in the other.

A second root counted for only 12 sections, in the center of the dividing area, was just as asymmetrical as the first. Thinking that this asymmetry might have been due to an asymmetrical exposure of the growing root to light, a root that had been grown in complete darkness was counted.

Table II shows the distribution of mitoses as noted in this root that had been grown in absolute darkness. It was just as asymmetrical for the 21 sections counted as eithef of the other two. When the halves 1 + 2 and 3 + 4 are compared 9 sections vary from symmetry by more than 10 per cent, the maximal variation being 24 per cent in one direction and 28 per cent in the other. When the halves 1 + 4 and 2 + 3 are compared 12 sections are more than 10 per cent asymmetrical, the maximal variation being 5 per cent in one direction and 54 per cent in the other. If sections containing only a few dividing cells, as at either end of the meristematic area, are included the percentage differences may be even higher.

On the basis of the observed asymmetry in normal onion roots by Rossmann, von Guttenberg, and ourselves we cannot help but agree with the first-mentioned workers and with Schwarz, that the existence of a

Table I

Series 38, sections cut 12 micra.

Sect. No.	No. Mitoses half 1, 2	Mitoses half 3, 4	Diff.	Diff.	Mitoses half 1, 4	Mitoses half 2, 3	Diff.	Diff.
				per cent				*per cent*
18	107	94	13	12	95	106	− 11	− 10
19	143	130	13	9	152	121	31	20
20	100	118	− 18	− 15	117	101	16	10
21	96	137	− 41	− 30	109	124	− 15	− 12
22	125	106	19	15	124	107	17	13
23	90	80	10	11	90	88	2	2
24	119	173	− 54	− 31	119	130	− 11	− 9
25	72	91	− 19	− 21	77	87	− 10	− 12
26	105	134	− 29	− 22	115	93	22	19
27	94	120	− 26	− 22	94	109	− 15	− 14
28	106	99	7	6	116	97	19	16
29	126	157	− 31	− 20	126	123	3	2
30	134	98	36	27	134	112	22	16
31	86	86	0	0	86	87	− 1	− 1
32	113	91	22	19	113	80	23	29
33	82	75	7	8	82	71	11	13
34	97	95	2	2	97	102	− 5	− 5
35	116	114	2	2	116	108	8	7
36	103	89	14	13	103	79	24	23
37	100	124	− 24	− 19	100	114	− 14	− 14
38	134	128	6	4	134	130	4	3
39	98	130	− 32	− 25	117	111	6	5
40	133	121	12	9	126	128	− 2	− 2
41	109	126	− 17	− 13	123	112	11	9
42	132	116	16	12	119	129	− 10	− 8
43	160	136	24	15	152	144	8	5
44	120	113	7	6	138	95	43	31
45	98	113	− 15	− 13	104	108	− 4	− 4
46	135	126	9	7	133	128	5	4
47	121	127	− 6	− 5	117	131	− 14	− 10
48	119	133	− 14	− 10	128	124	4	3
49	122	137	− 15	− 11	129	130	− 1	− 1
50	145	158	− 13	− 8	162	141	21	13
Totals:	3760	3876	− 116	− 3	3991	3645	346	9

mitogenetic radiation cannot be considered proved from the work done on onion roots.

Since it has been agreed that the M-rays are identical with ultraviolet light of a wave length of either 1900–2300 A° (Gurwitsch) or 3400 A° and 2800 A° (Reiter and Gabor), the rays should be able to affect a photograph plate, regardless of their intensity, if a long enough exposure was given, for the photographic plate is able to summate successive small amounts of light striking it. But although practically every worker in this field has tried to get such a photographic effect, all have failed in their attempts with the exception of Reiter and Gabor (1928) and these two workers suggest quite candidly that their results should be confirmed.

TABLE II

Series 152, sections cut 7 micra.

Sect. No.	No. Mitoses half 1, 2	Mitoses half 3, 4	Diff.	Diff. *per cent*	Mitoses half 1, 4	Mitoses half 2, 3	Diff.	Diff. *per cent*
59	74	70	4	5	70	74	− 4	− 5
60	22	29	− 7	− 24	35	16	19	54
61	32	23	9	28	33	22	11	33
62	41	31	10	24	41	31	10	24
63	35	37	− 2	5	38	34	4	10
64	52	49	3	6	57	44	13	23
65	40	36	4	10	42	34	8	19
66	43	51	− 8	16	46	48	− 2	− 4
67	56	54	2	4	55	55	0	0
68	53	44	9	17	51	46	5	10
69	72	62	10	14	66	68	− 2	− 3
70	76	61	15	19	67	70	− 3	− 4
71	78	56	22	28	67	67	0	0
72	85	80	5	6	92	73	19	21
73	81	73	8	9	86	68	18	21
74	66	61	5	7	70	58	12	17
75	75	71	4	5	81	65	16	20
76	66	61	5	7	73	54	19	26
77	80	86	− 6	7	95	72	13	14
78	55	57	− 2	4	68	44	24	35
79	94	114	− 20	18	106	102	4	4
Totals:	1276	1206	70	5	1339	1145	194	14

In an effort to confirm their results, and to establish on a purely physical basis the existence of a mitogenetic radiation, and if possible to determine more precisely the wave length of the M-ray, we have carried out three series of experiments in an attempt to obtain an effect on a photographic plate that could be ascribed to a mitogenetic radiation.

In the first experiments an effort was made to detect photographically an emission of M-rays from growing onion roots. The technical difficulties were considerable for the roots had to be kept moist while the photographic plate had to be kept absolutely dry to avoid the possibility of the so-called Russell effect.[1] Furthermore, while maintaining these conditions, the plate had to remain within a few centimeters of the roots for a considerable length of time.

To surmount these difficulties we placed a photographic film (Eastman Kodak Superspeed) inside of a quartz flask, which was blown especially for this work with a flat bottom only 0.2 mm. in thickness,[2] certainly thin enough not to offer any serious resistance to an ultra-violet radiation of 2000–2300 A°. While Schumann plate or film is more sensitive in the 2000 A° region (five times as sensitive to the 1850 line according to Adam Hilger, Ltd.) than ordinary film, Schumann film is notoriously unstable and it is doubtful if it could be used with such long exposures as are described later. Spectrograms of the Al spark taken with a Hilger quartz spectrograph on Kodak superspeed film (5 seconds) show the 1990, 1935, 1862 and 1854 lines although the last two are faint. Quartz test-tubes of 1.8 mm. wall thickness passed all the above lines, although there was undoubtedly considerable absorption of the last two but practically no absorption with 0.2 mm. thick quartz.

The film was pressed gently against the bottom, emulsion side down, with cotton or glass wool packing. The exposure was made by inverting the flask beneath the growing roots of an onion, the roots being allowed to grow down toward the flask from a distance of approximately three centimeters until they touched the upturned bottom of the flask, one half of which had been covered with a cover-glass to serve as control.[3] Negatives were exposed in this way to growing onion roots for 2½, 12½, and 24 hours. When developed, they showed no effects of a radiation of any sort.

These results are quite in keeping with those of other workers who have tried exposures of 48 hours' duration. To increase this exposure-time yeast cultures were substituted for the onion roots, Fleishman's yeast being used, and no attempt made to keep the culture pure.

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[1] The effect which various substances such as metals, cod liver oil, gelatin, gutta percha, celluloid, collodion, and certain vapors, etc., may exert upon a photographic plate through their oxygen-absorbing capacity. Hydrogen peroxide, one part in one million of water, will influence a photographic plate in eighteen hours. See Russell, W. J. (*Proc. Roy. Soc.*, London, 1899, **64**: 409–419, and *Photograph. Jour.*, 1899, **23**: 91–97), and Kugelmass and McQuarrie (*Science*, 1925, **62**: 87–88).

[2] Engineers of the General Electric Company estimate that quartz of this thickness will transmit 75 per cent of the ultra-violet light of 2000 angstroms wave length striking it. (Personal communication.)

[3] Which Rawin (1924) has found to be impenetrable to the M-rays.

For some of the experiments with yeast as a source of radiation, the quartz flask described above was used, the yeast culture being placed in the flask, and the flask being placed over a piece of cut film, one half of the area under the flask being covered with black paper to serve as a control. Care was always taken that the flask should not press directly against that area of the negative being exposed.

Exposures were made in this way for 13, 100, and 144 hours without affecting the exposed negative in any way. Microscopic examination of the yeast culture following, and during, each of the above exposures showed that in the medium used, Pasteur's with sugar, the budding activities of the yeast had just about stopped at the end of the five days, but that up to that time, budding plants in the culture were very numerous.

To obtain a still longer exposure to an actively budding yeast colony, the flask containing the culture was arranged as before over a negative, but this time the culture medium in the flask was renewed at intervals of from 2–3 days. This was done by merely pouring out the old medium and pouring in the new, enough yeast adhering to the flask to insure the proper inoculation of the new medium. After the solution had been changed the flask was returned to its original position over the negative, one half of the negative being covered to serve as a control to the other half. This experiment was set up in absolute darkness and remained in darkness for the whole length of the exposure, including the times when the change of culture medium was being effected.

In this way a negative was obtained that had been exposed to a constantly fresh and actively budding yeast colony for 15 days (360 hours), but even after this long an exposure the negative on being developed showed absolutely no effect from a radiation of any sort, in spite of the fact that an onion root exposed to such a culture for 20 minutes is supposed to show a marked mitogenetic effect from the exposure. Frank (1929) states that the onion root is 600 times more sensitive than the photographic plate. If an onion root can be affected in 20 minutes, a plate should be affected in approximately 600 × 20 minutes or 200 hours. In this experiment the plate was exposed for 360 hours.

The third series of experiments was set up in the following way. A series of quartz (1.1 to 1.8 mm. thick) and glass test-tubes, each containing a strip of cut film and each tightly stoppered to exclude all vapors, were partly immersed in bowls containing yeast culture. The upper part of each tube extended above the yeast culture so that in addition to the control negatives contained in the glass tubes, the upper part of each negative in the quartz tubes served as a control to the lower portion which extended into the yeast culture. The change of culture medium, made at intervals of 2–5 days, was easily and quickly accomplished by merely removing the tubes from the bowls containing the old medium and placing them in bowls containing the new. This series was set up and remained in complete darkness for the whole time the exposure was being made.

The negatives from the quartz tubes with their controls from the glass tubes were removed and developed simultaneously at intervals of 10, 40, 52, 80, and 89 days. But the negatives showed no effects from these long exposures to a supposedly potent source of mitogenetic radiation. A portion of each film used was exposed to light, and on development showed normal blacking, indicating that they were in good condition.

Discussion

If onion roots and yeast gave off a radiation of the nature of ultra-violet of an intensity sufficient to exert the potent physiological effects attributed to it, we believe that in 89 days it should have affected in a noticeable manner a photographic plate exposed to them. In view of the fact that such an effect was not obtainable, we cannot help but conclude that these tissues do not give off such a radiation, and that if the tissues exert a mitogenetic effect it must be through some agency other than the emission of ultra-violet waves.

These experiments do not, of course, exclude the possibility that exposure of cells to minimal amounts of ultra-violet light may stimulate cell division. They make it practically certain, however, that budding yeast and onion root tips produce no ultra-violet radiation of 2000 A° or longer. Indeed, the emission of such a radiation from a living cell would be extremely unlikely. Even luminous animals, which produce visible radiation, have a spectrum which stops far short of the ultra-violet. Experiments with *Cypridina* luminescence whose spectral maximum lies at $\lambda = .48\mu$ (Coblentz, 1926) show that this light produces exactly the same effect on a photographic plate when exposed through quartz as when exposed through glass. No difference in density after development could be detected between the quartz- and glass-protected regions from exposures not long enough to give maximum blackening, *i.e.*, care was taken to make the exposure correspond to the region of the plate where increased exposure gives increased blackening.[4]

No chemical reactions in aqueous media have ever been definitely shown to emit ultra-violet light. The alleged effects of this kind (Matuschek and Nenning, 1912) have been found to be due to the action of vapors (Mathews and Dewey, 1913), an effect against which too great precautions cannot be taken.

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[4] Harvey, E. N., unpublished experiments carried out in 1925.

We believe that a root-tip, in which cell division is observed to be far from uniform, is unfortunate material to work with. Those who have studied yeast know the difficulties of estimating budding under different conditions. Indeed, if the division or budding of one cell can affect the division, or budding, of another, we should find the growth of organisms to increase more rapidly than corresponds to logarithmic increase. Rate of cell division should be dependent on volume of suspended organisms. No such effect has been recognized. The allelocatalytic phenomenon of Robertson has not been generally confirmed by other workers. (Richards, O. W., Thesis.) The alleged mitogenetic effect could be due to insufficient control of experimental material, primarily difficult to work with. For this reason and in view of the alleged effects from material containing no dividing cells, hemolysed blood, contracting muscle, conducting nerves, etc., we are inclined to place them in the same category as the famous n-ray [5] of Blondlot (1903), shown to be a purely subjective phenomenon by Wood (1904) and Gehrcke (1905).

Conclusions

1. Evidence is offered to show that the onion root as a detector of the mitogenetic rays cannot be relied on, since in normal roots, unexposed to any supposed source of mitogenetic radiation, there may still be a variation in the number of dividing cells in the two halves of a root as high as 50 per cent.

2. Exposure of a photographic plate to growing onion roots through .2 mm. quartz for 48 hours failed to detect a mitogenetic radiation.

3. Exposure of photographic negatives to an actively growing and dividing yeast culture through quartz 1.1 mm. thick for as long as 89 days failed to affect the negative in any way.

4. The authors conclude in view of their negative evidence that the existence of a mitogenetic radiation in the form of ultra-violet light by normally growing onion roots and yeast plants cannot be accepted as a fact.

BIBLIOGRAPHY

Aniken, A. W., 1926. Das Nervensystem als Quelle mitogenetischer Strahlung. *Arch. f. entw.-Organism.*, **108**: 609.

Baron, M. A., 1926. Über mitogenetische Strahlung bei Protisten. *Arch. f. entw.-Organism.*, **108**: 619.

Baron, M. A., 1928. Bakterien als Quellen mitogenetischer (ultravioletter) Strahlung. *Centralbl. f. Bakt., Parasit. u. Infekt.*, Abt. 2, **73**: 373.

Blacher, L. J., 1930. Die Rolle der mitogenetischen Strahlungen in den Prozessen der Metamorphase der Schwanzlosen Amphibien. *Arch. f. entw.-Organism.*, **122**: 48.

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[5] For further literature on this subject see Charpentier, Lambert and Meyers in the *Compt. rend. Soc. Biol.* (Paris) for 1904–05.

Blacher, L. J., and N. W. Bromley, 1930. Mitogenetische Ausstrahlungen bei der Schwanzregeneration der Urodelen. *Arch. f. entw.-Organism.*, **123**: 240.

Blacher, L. J., and O. G. Holzmann, 1930. Resorptionsprozesse als Quelle der Formbildung. III. Mitogenetische Ausstrahlungen während der metamorphose bei Urodela. *Arch. f. entw.-Organism.*, **123**: 230.

Blondlot, R., 1903. Sur la propriété d'émettre des rayons n, que la compression confère à certains corps, et sur l'émission spontanée et indéfinie de rayons n par l'acier trempé, le verre trempé, et d'autres corps en état d'équilibre moléculaire contraint. *Compt. rend. Acad. Sci.*, **137**: 962.

Borodin, D. N., 1930. Energy Emanation during Cell Division Processes (M-Rays). *Plant Physiol.*, **5**: 119.

Bromley, N. W., 1930. Der Einfluss der primären Verheilung der Wunde auf die Entstehung mitogenetischer Ausstrahlungen in ihr. *Arch. f. entw.-Organism.*, **123**: 274.

Choucroun, N., 1930. On the Hypothesis of Mitogenetic Radiation. *Jour. Mar. Biol. Assn., Plymouth*, **17**: 65.

Coblentz, W. W., and C. W. Hughes, 1926. Spectral Energy Distribution of the Light Emitted by Plants and Animals. *Bur. Stand. Sci. Papers*, **21**: 521.

Frank, G., 1929. Das mitogenetische Reizminimum und -maximum und die Wellenlänge mitogenetischer Strahlen. *Biol. Zentralbl.*, **49**: 129.

Frank, G. M., and A. Gurwitsch, 1927. Zur Frage der Identität mitogenetischer und ultravioletter Strahlen. *Arch. f. entw.-Organism.*, **109**: 451.

Frank, G., and M. Kurepina, 1930. Die gegenseitige Beeinflussung der Seeigeleier als mitogenetischer Effekt betrachtet. *Arch. f. entw.-Organism.*, **121**: 634.

Frank, G., and M. Popoff, 1929. Le rayonnement mitogénétique du muscle en contraction. *Compt. rend. Acad. Sci.*, **188**: 1010.

Frank, G., and S. Salkind, 1926. Die Quellen der mitogenetischen Strahlung im Pflanzenkeimling. *Arch. f. entw.-Organism.*, **108**: 596.

Frank, G., and S. Salkind, 1927. Die mitogenetische Strahlung der Seeigeleier. *Arch. f. entw.-Organism.*, **110**: 626.

Gehrcke, E., 1905. Zur Deutung der Versucke mit N-Strahlen. *Physik. Zeitschr.*, **6**: 7.

Gesenius, H., 1930. Über die Gurwitschstrahlung menschlichen Blutes und ihre Bedeutung für die Carcinomdiagnostik. *Biochem. Zeitschr.*, **226**: 257.

Gurwitsch, A., 1923. Die Natur des spezifischen Erregers der Zellteilung. *Arch. f. entw. Mech.*, **100**: 11.

Gurwitsch, A., 1924. Les problêmes de la mitose et les rayons mitogénétiques. *Bull. d. Histol. Appl.*, **1**: 486.

Gurwitsch, A., 1926. Das Problem der Zellteilung physiologisch betrachtet. Monogr. Gesamtgeb. Physiol. Pflanz. u. Tiere, No. 2. Springer, Berlin.

Gurwitsch, A., 1928*a*. Some Problems of Mitogenetic Rays. (Russian paper with German summary.) *Jour. Soc. Bot. Russie Acad. Sci.*, **13**: 179.

Gurwitsch, A., 1928*b*. Einige Bemerkungen zur vorangehenden Arbeit von Herrn B. Rossmann. *Arch. f. entw.-Organism.*, **113**: 406.

Gurwitsch, A., 1929. Über den derzeitigen Stand des Problems der mitogenetischen Strahlung. *Protoplasma*, **6**: 449.

Gurwitsch, A., and L. Gurwitsch, 1925*a*. Weitere Untersuchungen über mitogenetische Strahlungen. *Arch. f. mikr. Anat.*, **104**: 109.

Gurwitsch, A., and L. Gurwitsch, 1925*b*. Über den Ursprung der mitogenetischen Strahlen. *Arch. f. entw.-Organism.*, **105**: 470.

Gurwitsch, A., and L. Gurwitsch, 1926. Die Produktion mitogener Stoffe im erwachsenen Tierischen Organismus. *Arch. f. entw.-Organism.*, **107**: 829.

Gurwitsch, A., and L. Gurwitsch, 1928. Über ultraviolette Chemolumineszenz der Zellen im Zusammenhang mit dem Problem des Carcinoms. *Biochem. Zeitschr.*, **196**: 257.

Gurwitsch, A., and N. Gurwitsch, 1924. Fortegesetzte Untersuchungen über mitogenetische Strahlung und Induktion. *Arch. f. entw.-Organism.*, **103**: 68.

Gurwitsch, L., and A. Anikin, 1928. Das Cornealepithel als Detektor und Sender mitogenetischer Strahlung. *Arch. f. entw.-Organism.*, **113**: 731.

Gurwitsch, A., and G. Franck. Sur les rayons mitogénétiques et leur identité avec les rayons ultraviolets. *Compt. rend. Acad. Sci.*, **184**: 903.

Guttenberg, H. von, 1928*a*. Die Theorie der mitogenetischen Strahlen. *Biol. Zentralbl.*, **48**: 31.

Guttenberg, H. von, 1928*b*. Schlusswort zur Arbeit von B. Rossmann. *Arch. f. entw.-Organism.*, **113**: 414.

Hollaender, A., and E. Schoeffel, 1931. Mitogenetic Rays. *Quart. Rev. Biol.*, **6**: 215.

Holzmann, O. G., 1930. Mitogenetische Ausstrahlungen während der Metamorphose bei Drosophila melanogaster. *Arch. f. entw.-Organism.*, **123**: 266.

Kisliak-Stratkewitsch, M., 1927. Das mitogenetische Strahlungsvermögen des Kartoffelleptoms. *Arch. f. entw.-Organism.*, **109**: 283.

Magrou, J., and M. Magrou, 1927. Recherches sur les radiations mitogénétiques. *Bull. d'Histol. Appl.*, **4**: 253.

Magrou, J., and M. Magrou, 1928. Action à distance du Bacterium tumefaciens sur le développement de l'oeuf d'Oursin. *Compt. rend. Acad. Sci.*, **186**: 802.

Magrou, J., M. Magrou, and F. Choucroun, 1929. Action à distance du Bacterium tumefaciens sur le développement de l'oeuf d'Oursin (nouvelles expériences). *Compt. rend. Acad. Sci.*, **188**: 733.

Mathews, J. H., and L. H. Dewey, 1913. The Production of Photochemically Active Rays in Ordinary Chemical Reactions. *Jour. Phys. Chem.*, **17**: 230.

Matuschek and Nenning, 1912. Ueber das Auftreten von chemisch wirksamen Strahlung bei chemischen Reaktionen. *Chem. Zeitung*, **36**: 21.

Potozky, A., and Q. Zogline, 1929. Untersuchungen uber die mitogenetische Strahlung des Blutes. *Biochem. Zeitschr.*, **211**: 352.

Rawin, W., 1924. Weitere Beitrage zur Kenntnis der mitotischen Ausstrahlung und Induktion. *Arch. f. mikr. Anat.*, **101**: 53.

Reiter, T., und D. Gabor, 1928. Zellteilung und Strahlung. Springer, Berlin.

Rossmann, B., 1928. Untersuchungen über die Theorie der mitogenetischen Strahlen. *Arch. f. entw.-Organism.*, **113**: 346.

Rossmann, B., 1928-29. Mitogenetische Inductionsversuche mit Hefe als Indicator. *Arch. f. entw.-Organism.*, **114**: 583.

Rusinoff, P. G., 1925. Weitere untersuchungen über mitogenetische Strahlen und Induktion. *Arch. f. mikr. Anat.*, **104**: 121.

Salkind, S., 1929. Über den Rhythmus der mitogenetischen Strahlung bei der Entwicklung des Seeigeleies. *Arch. f. entw.-Organism.*, **115**: 360.

Salkind, Potozky, und Zoglina, 1930. Die mitogenetische Beeinflussung der Eier von Protodrilus und Saccocirrus. *Arch. f. entw.-Organism.*, **121**: 630.

Schwarz, Walter, 1928. Das Problem der mitogenetischen Strahlen. *Biol. Zentralbl.*, **48**: 302.

Siebert, W. W., 1928*a*. Über die mitogenetische Strahlung des Arbeitsmuskels und einiger anderer Gewebe. *Biochem. Zeitschr.*, **202**: 115.

Siebert, W. W., 1928*b*. Über die Ursachen der mitogenetischen Strahlung. *Biochem. Zeitschr.*, **202**: 123.

Siebert, W. W., 1930. Die mitogenetische Strahlung des Blutes und des Harns gesunder und kranker Menschen. *Biochem. Zeitschr.,* **226**: 253.

Sorin, A., 1926. Zur Analyse der mitogenetischen Induction des Blutes. *Arch. f. entw.-Organism.,* **108**: 634.

Sorin, A. N., 1928. Über mitogenetische Induction in fruheren Entwichlungsstadien des Huhnerembryo. *Arch. f. entw.-Organism.,* **113**: 724.

Stempell, W., 1929. Die Lebensstrahlen. *Strahlentheraphie,* **34**: 868.

Urbanowicz, K., 1927. Gurwitsch's mitogenetische Strahlung, an Paramäzienteilungen Geprüft. *Arch. f. entw.-Organism.,* **110**: 417.

Wagner, N., 1927. Über den von A. Gurwitsch entdeckten spezifischen Erreger der Zellteilung (Mitogenetische Strahlen). *Biol. Zentralbl.,* **47**: 670.

Wassiliew, L. L., G. M. Frank, und E. E. Goldenberg, 1931. Versuche über die mitogenetische Strahlung des Nerven. *Biol. Zentralbl.,* **51**: 225.

Wood, R. W., 1904. Die N-Strahlen. *Physik. Zeitschr.,* **5**: 789.

SOME OBSERVATIONS ON THE EGGS OF FUCUS AND UPON THEIR MUTUAL INFLUENCE IN THE DETERMINATION OF THE DEVELOPMENTAL AXIS

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Several species of the seaweed *Fucus* are obtainable in abundance on the rocky shores of Nahant peninsula, outside the entrance to Boston Harbor. These seaweeds have a long breeding season, shedding eggs and sperm in the winter months when other types of eggs are difficult to obtain. In 1929–30 eggs of *Fucus vesiculosus* were collected from October until June. Throughout this time some eggs were always obtainable from plants with relatively large fruiting tips, or receptacles, and in February, March, April, and May they were obtainable in great abundance.

Fucus vesiculosus is dioecious. The sexes can be separated by sight with fair reliability if a cut receptacle is examined. The conceptacles of the male plants are orange due to the carotinoids contained in the antherozoid or sperm cells. The conceptacles of the female plants are green or brownish-green, largely because of the plastids in the eggs. Identification becomes certain if a thin section of the receptacle is examined microscopically.

The purpose of this paper is to present a number of observations and experiments, some of which have been incidental to measurements on respiration in the *Fucus* eggs which will be presented elsewhere. The results recorded here have particularly to do with the nature of the eggs and with certain factors involved in determining the first division plane. The first division of the *Fucus* egg ordinarily gives rise to two cells of different shape. One, which includes the rhyzoidal protuberance, is the parent cell for the formation of the rhyzoid, the other gives rise by divisions to the thallus. At the first division, therefore, the polarity or developmental axis of the spore has been determined and is first indicated. A number of environmental factors have been found capable of determining the cleavage plane and the polarity of the *Fucus* spore. Orientation of the cleavage by a directed beam of light has been demonstrated in a number of plants (*e.g.,* Pierce, 1906). Farmer and Williams (1898) have shown that if fertilized *Fucus* eggs are illuminated from one side, the rhyzoids usually originate on the side of the egg remote from the light. Miss Hurd (1920) found that in *F. inflatus* (a monœcious species, collected in San Francisco Bay) when directed beams of different light frequencies are used, red light has no effect but the short blue orients the cleavage and the direction of growth of the rhyzoid.

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At Friday Harbor Lund (1923) passed an electric current through sea water containing spores of the monoecious *Fucus inflatus.* Cross streams of sea water were designed to carry off electrolytic products formed at the electrodes. A potential drop of 25 millivolts across the diameter of an egg oriented the division plane and the developmental axis. The rhyzoid cell came to lie toward the + pole.

There are no visible marks or identifications of polarity in the unfertilized *Fucus* egg. The nucleus lies in the middle of the cell. When spores which are not too greatly crowded develop in the dark, the directions of divisions lie entirely at random. Miss Hurd (1920) observed in her work with colored light that if the eggs lie close together, within 2 or 3 egg diameters, they tend to send out the rhyzoid toward each other, or toward the center of a nearby mass of eggs. This phenomenon, which she calls a "group effect," was especially pronounced in the dark, but was strong enough to overcome the orienting effect of the directed light if the eggs were close together.

This directive effect of one egg on another at a distance presents some points of interest. It might possibly be due to a differential of oxygen tension, or of CO_2, or to the accumulation of some other metabolite. If the cells are either giving off or consuming some substances in radial fashion, in the case of neighbors the additive effect on the intervening space between them would cause that part of the sea water environment to be most altered. No jelly or solid substance of the eggs traverses this space. Winkler (1900) attempted to establish a gradient of oxygen tension across the spores of *Cystosira barbata,* which are also oriented by light, to see if this might be the determining factor. The results were negative. In view of recent work purporting to show an effect at a distance upon dividing cells due to "mitogenetic" radiation, the possibility of some such effect in the "group effect" in *Fucus* presents itself. Mitogenetic rays have been supposed to affect primarily the division rate of neighboring cells, rather than the polarity or plane of division. It is possible that this qualitative distinction is not justified, however, as the plane of division may be determined by asymmetric or differential rates of the processes leading to cell division. The experiments which are to be described do not discover the nature of this influence of cells at a distance. It is at present possible to answer the following two questions: 1. In order that cells shall exert this influence upon a neighbor, must there be nuclear activity or cell division in the directing cells? 2. Is the directive effect in *Fucus* specific? The answers to these questions still leave open the question of a possible rôle of mitogenetic rays, since this type of radiation has been as well demonstrated coming from non-dividing tissues, even recently macerated tissue, as from dividing cells. A brief review of some of this work is given by Hollaender and Schoeffel, 1931. Further experiments to continue these preliminary observations are planned.

Before proceeding to a consideration of these experiments, however, a description of the *Fucus* eggs, and the results of some other observations, will be presented.

The Gametes of Fucus

The *Fucus* plant is diploid. As in animals the haploid generation is confined to the gametes. Yamanouchi (1909) has estimated the chromosome number in *F. vesiculosus* to be 64–32. After the plants had been brought into the laboratory the sex of each plant was determined by microscopical examination of a section of a receptacle. The receptacles were then cut off and placed in covered glass dishes in an ice box at about 3° C. Usually within 24 hours or less the gametes within their capsules had been extruded from the conceptacles to lie in mounds on the outside of the receptacles. Removal of the plants from sea water induces shedding. In nature the shedding is stimulated when the plants are stranded at low tide. Too much drying, however, is highly detrimental, and it is best to keep the receptacles in covered dishes as well as to keep them cool.

The eggs lie 8 in a capsule when shed. The capsule wall consists of two thin membranes, probably with a gelatinous substance between, 10 microns wide. When $KI + I_2$ is added, and then H_2SO_4, the capsule membranes and the space between them do not turn blue (although the eggs within do). This indicates that the capsule is not made of cellulose. When HNO_3 and NH_3 are added these membranes, and especially the space between them, turn deep yellow-brown, indicating that they are proteinaceous. The membranes coagulate and partly dissolve when the acid is added, and some coagulation of the substance between the membranes is observed. More slowly the eggs also turn yellow, although the color of the plastids tends to some extent to obscure the color.

When the egg capsules are washed off from the fruiting tips into normal sea water, after a time, depending on temperature, first the outer and later the inner membrane of the capsule breaks and the eggs are aborted into the sea water, often being somewhat squeezed together in passing out. If the eggs are kept cold, they apparently undergo no deterioration for many days. High percentages of fertilization and of normal spores have been obtained from eggs which had remained unfertilized, either in light or dark, for more than a week.

Many diflagellate sperm or antherozoids occur in small capsules (64 antherozoids per capsule, according to Yamanouchi, 1909). These capsules are exceedingly delicate and appear to dissolve completely in the sea water. The sperm are immotile when liberated, but they begin to move in a few seconds, and are soon swimming actively. My own experience has been that their life is short, or rather that they do not fertilize eggs well after a few hours, especially if they have been in dilute suspension. They may be kept a number of days, however, if they remain dry on the fruiting tips in a moist chamber. They are immotile in this condition. Sperm suspensions of even moderate concentration are brilliant orange.

The unfertilized eggs of *Fucus vesiculosus* vary considerably in size. Single eggs varied between 52 and 70 microns in diameter, averaging between 60 and 65. Larger eggs are found which will be discussed later. The eggs are readily stratified by centrifuging, and develop normally after stratification. Eggs centrifuged immediately after fertilization tend to become amœboid but eventually round up. Centrifuging for 20 minutes at 2600 r.p.m. (20 cm. radius, 18° C.) throws almost all of the formed bodies to one end. A gray cap occupies the end position. Its thickness is about one ninth the diameter of the egg. The nucleus lies at its inner edge, just between it and the plastids, which are densely crowded into a zone bordering the gray cap. A few plastids remain behind adhering to the peripheral regions of the egg. The stratified materials are all less dense than the cytoplasm at large, as the gray cap is seen to float uppermost when the eggs settle in a tube and are observed from the side as they fall. The eggs are comparatively dense. They settle more rapidly than the eggs of such animals as *Arbacia, Cumingia, Chætopterus,* etc., in spite of their smaller size. The volume of materials which are moved by the centrifuge is much less in proportion than in such animal eggs as the sea urchin's. Since the eggs are photosynthetic they are perhaps able to dispense with as large a store of food materials.

The unfertilized eggs may readily be cut with the microneedle, although unless they are pinched gently with care, they burst and disperse their substance into the sea water. They appear to be only poorly prepared to gelate a new surface on an exposed cut, being deficient in materials for what Heilbrunn has called the "surface precipitation reaction."

Fertilization

When a heavy sperm suspension is added to unfertilized eggs, the eggs are rapidly rotated by the sperm. After a few minutes they stop rotating even though the sperm are still fully active. That this whirling of the eggs has no significance in bringing about fertilization is evident (1) because dilute sperm suspensions which fertilize the eggs cause no rotation, and (2) because concentrated sperm of another member of the *Fucaceæ, Ascophyllum nodosum,* which do not fertilize the eggs of *Fucus vesiculosus* rotate the *Fucus* eggs more rapidly than the eggs of their own species (which are larger).

Fertilized eggs were placed in a thermostat at 18° C., and the time-lapse to the first division was noted. The time span over which first divisions occur in a population is a wide one, covering approximately 13 to 18 hours. Fifty per cent of the eggs were observed to have divided after 14 hours in one case, and after 15 hours in another. At cold temperatures the time is greatly extended.

The *Fucus* egg has been described as secreting a cellulose wall immediately after fertilization. Eggs were fertilized and then cut with the microneedle at increasing intervals after fertilization, to determine the physical nature of this secretion and the sequence of physical change. It has been stated that the unfertilized eggs may readily be cut in half, although they burst very easily. When they burst no membrane of any sort is visible around the egg or remaining behind. Five minutes after fertilization the eggs may be cut with the greatest ease. There is no longer any tendency to burst. When the fragments are separated with the needle a sticky gelatinous material, which has been secreted, is seen to bridge across between the fragments. At 10 minutes conditions are about the same. Fifteen minutes after fertilization a slight increase in the rigidity of this gelatinous secretion is noticeable. After 30 minutes it is slightly more firm. At 45 minutes it has become a definite semi-solid wall which holds its shape but which is still easily cut. After an hour the secretion has become so firm as to be cut only with great difficulty, and usually only after bursting the egg within. After an hour and a half the wall is tough and rigid. The eggs dodge the needle and cannot be cut.

It is probably this sticky secretion which causes the eggs to adhere to the substrate and stop rotating after fertilization. The eggs adhere to the substrate with increasing tenacity until by 2½ hours they are fairly well attached, or if they are kept in suspension by gentle shaking, after 2½ hours they are clumped together in adhering masses. After the secreted wall has become firm the eggs are well protected and may at any time be dislodged without damage from the substrate to which they adhere.

The secreted wall of the fertilized egg was subjected to colorimetric cellulose and protein tests. The walls of 24-hour spores gave no protein reaction, although of course the egg itself and cytolytic extrusions from it turned deep yellow. The cell walls turned brilliant blue when subjected to $KI + I_2 + H_2SO_4$, a test for cellulose. No rotation of polarized light was observed with a polarizing microscope, but this may well have been because the cellulose is too thin to cause a detectable amount of rotation.

The unfertilized eggs show a faint blue color in the cellulose test, perhaps due to the material in the cortex which is to be secreted. Ten minutes after fertilization the color reaction of the secreted jelly is equally faint. At 25 and 55 minutes there is a slight increase in the blue reaction. At about an hour and a half the first decidedly blue color results. Even this, however, is not as brilliant a blue as in the 24-hour eggs. It seems probable that a relative of cellulose is secreted as the sticky jelly, and that gradually the crystal molecular arrangement is assumed so that by an hour and a half the tough cellulose wall has been formed, which gives the blue color reaction. As the rhyzoid grows out on one side this membrane must be softened and added to; at any rate it precedes and sheathes the early rhyzoid. Nevertheless it gives a brilliant blue color reaction at the tip as if it does not reconvert back into the faintly coloring jelly.

If eggs which have been fertilized for an hour and a half are placed in sea water saturated with dextrose they shrink and collapse. Unfertilized eggs do not collapse. Eggs which have been fertilized for 25 minutes do not collapse, while those which have been fertilized for 45 minutes collapse to some extent. Eggs which have been fertilized for 3 hours or longer collapse and leave behind the transparent cellulose wall, which is clearly revealed in this way. Farmer and Williams note that placing the eggs in tap water so that they burst and flow out also reveals the cell wall. The difference in behavior in dextrose sea water is often a convenient and fairly reliable way of testing for fertilization, as the jelly and the cellulose wall are not themselves readily seen in the normal fertilized egg. After two and a half hours in dextrose-saturated sea water the eggs have mostly rounded out and recovered their shape. Shrinking with sugar in the later stages causes the individual cells of the spore to stand out clearly. Even the early spores have a remarkable ability to withstand dehydration and to develop normally after being returned to normal sea water. Thus embryos one hour, an hour and a half, and two hours after fertilization were placed in dextrose-saturated sea water for two hours. They were then returned to normal sea water, and developed in typical fashion.

The Origin and Fate of Giant Eggs

The size of single eggs of *Fucus vesiculosis* eggs varies considerably (about 52–70 microns). In addition, a number of giant eggs, often much larger, are frequently found in a sample. The proportion of these giants depends greatly on the treatment to which the eggs have been subjected before their emergence from the capsules. In some cases more than half will be giants, most of which are much larger than the eggs seen within the capsule. This difference in size led Behrens, 1886 (cited by Farmer and Williams, 1896), to propose that the large eggs represent a stage in fertilization. Farmer and Williams (1896, 1898) point out that by no means are all fertilized eggs larger than the unfertilized eggs in the capsules, and they further noted that two or three nuclei are sometimes visible in these large eggs, which they therefore regarded as abnormal eggs. Examples of what appear to be giant eggs appear in photographs by Hurd (1920) and Lund (1923). It might be supposed that these large multinucleate eggs result from the failure of certain of the parent germ cells to divide. While this may possibly be the case sometimes, careful direct observations have shown another and simple origin of frequent occurrence.

When the eggs come out of the capsule they are often pressed together. At this time they often fuse to form giants. These giants have been observed to form as the result of the fusion of 2, 3, 4, 5, 6, 7, and 8 single eggs. The giant egg, of course, contains the corresponding number of nuclei, although possibly these fuse later. Once eggs have fused, they have never been seen to separate later. The effect of temperature at the time of emergence is very marked. Samples of the same set of capsules were divided into lots, some of which were placed at 3° C. and some at between 25° C. and 28° C., during the period of break-down of the capsules and release of the eggs. At the lower temperature the break-down of the capsules takes a much longer time, but after the eggs were out this striking difference was found: In the eggs at 3° C. only 3 giants were found in about 6000 eggs. In the eggs which had emerged at 25–28° C. between 50 per cent and 60 per cent of all the eggs were giants, mostly of about 4 fused eggs. The high temperature apparently alters what might loosely be called the consistency of the eggs, so that they are much more prone to fuse. One of the benefits of keeping the eggs cold while they are emerging from the capsules is to avoid these giants in experiments in which they are to be avoided. The plants should be kept cold from the time of collecting, as otherwise some fusions will take place within the capsules.

The development of these giants involves some peculiarities and variations. No doubt they occur to some extent in nature when the eggs are shed on warm days. Many hundreds of individual eggs were isolated and fertilized, and single mononucleate eggs were never observed to undergo abnormal cell division nor to give rise to more than one rhyzoid (although this in some cases branched at a later time). The giants also usually divided in normal fashion, with one giant rhyzoid, but not infrequently two and sometimes three independent rhyzoids developed simultaneously from various parts of the egg. The number of rhyzoids bore no special relation to the number of cells which had fused to form the giant egg, except that there were never more rhyzoids than component cells. Thus, isolated giants which had been seen to originate from the fusion of six eggs (for example) formed in some cases one, in others two, and occasionally three rhyzoids. Most commonly when two rhyzoids formed they grew out side by side, sometimes having a common basal part. Not infrequently, however, when reared in the dark and away from neighbors, two rhyzoids grew out 180° or less apart. A few examples are sketched in Fig. 1.

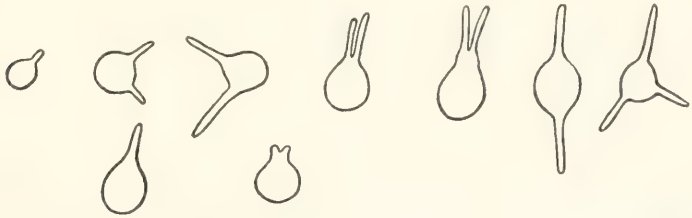

FIG. 1. Spores from fused eggs (*F. vesiculosis*). The small left-hand spore is a single egg.

Sometimes one of the supernumerary rhyzoids, especially if it is small, may disintegrate and sluff off, while the remainder of the spore then develops normally.

The causes which determine whether twinning, or what degree of twinning, shall take place in these giant spores are not known. Two possibilities suggest themselves. It may depend upon how many of the constituent egg cells receive sperm independently, or it might depend upon the degree of fusion of the constituent cells, as determining whether the egg nuclei may come together to form a single polyploid nucleus, or whether internal partitions (former cell surfaces) persist, holding the nuclei apart so that they become independent division-centers. At any rate, the relation between the twinning and the fused origin of the giant eggs indicates that the phenomenon is of the same general sort as the duplication in parts which results from experimentally fused eggs or blastulæ of sea urchins and starfishes (cf. Morgan's text, 1928).

Cross-Fertilization

In the spring gametes were obtained from the monoecious *Fucus evenescens*.[2] The eggs and sperm are shed simultaneously in a heavy mass of viscous jelly which covers the entire fruiting tip. These eggs are larger than those of *F. vesiculosus,* averaging about 85–90 microns in diameter. They also differ in color, being reddish-brown instead of brownish-green. The immature eggs of *F. evenescens* within the conceptacles are quite green, indicating that the reddish-brown pigments develop in the eggs as they approach maturity. Later in the spring (in April and May) gametes from the dioecious *Ascophyllum nodosum* were also obtained in abundance. The eggs of this form are green. They vary in diameter about between 60 and 85 microns, and occur four in a capsule instead of eight.

Experiments were designed to see if cross-fertilizations will take place among these three species. Fruiting tips were washed in fresh water to kill any antherozoids which might be upon them, and were dried with filter paper and then dipped in filtered sea water to restore the normal salt environment. They were then placed in individual moist chambers to shed.

Eggs and sperm from a given fruiting tip of *F. evenescens* often exhibited 99 per cent fertilization. It is apparently not necessary for the sperm of different individuals to be exchanged for the highest percentages of fertilization, as in some hermaphroditic animals such as the ascidian *Ciona* (Morgan, 1924).

Cross-fertilizations were attempted as follows: *F. vesiculosus* sperm × *Ascophyllum* eggs, *F. vesiculosus* eggs × *Ascophyllum* sperm, *F. vesiculosus* eggs × *F. evenescens* sperm. In all cases samples of the eggs and sperm used were also tested against the corresponding gametes of the same species as a control to be sure that they were in good condition. Samples of *F. vesiculosus* and *Ascophyllum* eggs were also run as unfertilized controls to check against contamination. Since *F. evenescens* eggs could not be obtained separately from sperm no attempts were made to fertilize them with foreign sperm. The *F. evenescens* sperm could be obtained free from eggs by drawing off sea water above *evenescens* eggs which had settled in a dish. *F. vesiculosus* sperm were added to *Ascophyllum* eggs in four experiments. In the first experiment about ten per cent of the eggs divided, but also about ten per cent of the unfertilized controls developed. Either contamination or parthenogenesis occurred. The experiment was repeated three more times with large dishes of eggs with no further development whatsoever. The controls were good in these experiments. *Ascophyllum* sperm were added to *F. vesiculosus* eggs in three experiments. In two cases no divisions took place. In the third case three eggs in about 3,000 divided. The controls were all good. *F. vesiculosus* eggs were inseminated with *F. evenescens* sperm in ten experiments. The controls were all good. The percentages of development in the ten experiments were as follows: 1 per cent, 1 per cent, 0.1 per cent, 0, 0, 0, 0, 0, 0, 0.

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[2] I am indebted to Professor W. R. Taylor for the identification of this species from a pressed specimen.

The conclusion must be that less than 1 per cent cross-fertilization takes place among these forms in normal sea water.

The "Group Effect"

In the course of some other experiments large numbers of dishes of *F. vesiculosus* eggs had been reared in the dark and many examples were seen of what appeared to be division of eggs so that the rhyzoids pointed toward near neighboring spores. Since it would not be difficult to be deceived, with neighboring cells in all directions, some sixty dishes were prepared in which only two eggs were placed between one and three egg-diameters apart. These were reared in the dark and were later examined microscopically with an apparatus which made it unnecessary to jar or touch the dishes. The results were then tabulated, dividing the circle around each egg into quadrants, one of which included the angle 45° to either side of the line joining the two eggs. Counts were made of the eggs whose rhyzoids protruded in each quadrant. The count showed an entirely random distribution. There was no correlation between the division plane and the position of the neighboring cell. This result was very surprising as some very convincing signs of correlation had been seen in dishes containing many cells. Accordingly, dishes were prepared in which a large compact mass of eggs lay in the center of the dish, and around about the periphery of the mass isolated eggs were placed at intervals well apart and about two egg diameters out from the peripheral cells of the mass. In this configuration there is no ambiguity as to the direction of the neighboring cell mass as in a random scattering over the bottom of a dish. The eggs were reared in the dark. The results in these cases were as clear-cut as in those with only two eggs in a dish, but they were quite the opposite. Some actual counts of the directions in which the rhyzoids protruded from these outlying cells are as follows: Within the 180° of arc toward the central mass 257 eggs, in the 180° away from the mass 2 eggs; in 57 eggs, out

1 egg; in 340, out 7; in 40, out 3 etc. The peripheral cells of the central mass itself also divided with rhyzoids inward in comparable proportions. It appears then that some general condition resulting from large numbers of eggs in the dish is necessary in order that several neighbors shall have this mutual effect. The causes of this were not worked out. For the present purpose it was sufficient to find a circumstance in which the "group effect" invariably takes place. This condition is met when eggs are placed around a central mass of hundreds or thousands of eggs in a Syracuse dish, regardless of the shape of the mass. In very small dishes a smaller mass may suffice.

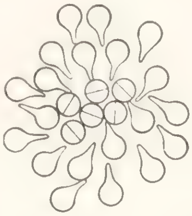

FIG. 2. Outline sketch of a group of *F. evenescens* eggs reared in the dark, showing the "group effect." Some of the inner cells have divided equally without producing rhyzoids.

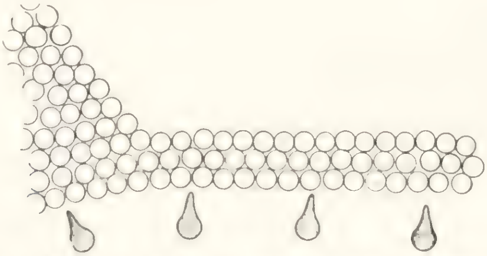

FIG. 3. Sketch of part of an experiment, showing *F. evenescens* spores (stippled) directed by unfertilized *F. vesiculosus* eggs. These spores were reared in the dark, and *F. evenescens* eggs are on one side only of the *F. vesiculosus* mass.

The eggs of *Ascophyllum* also exhibit the "group effect" and the eggs of *F. evenescens* show it very markedly, much more markedly than the eggs of *F. vesiculosus*. That is to say, even small isolated groups of these eggs alone in a dish develop rhyzoids pointing toward the nearest neighbor, or in the resultant direction if there are a number of neighbors. Rings of four or five eggs have the rhyzoids all pointing inward. In large masses not only the peripheral eggs have rhyzoids pointing inward, but often five or six outer layers of cells also all point inward. In masses of eggs of this species, especially larger masses, the innermost cells not infrequently divide into two equal and similar cells instead of producing one rhyzoid cell. Farmer and Williams observed occasional equal divisions in *F. vesiculosus* eggs, especially when reared in the dark. Some actual counts of the direction of the rhyzoid in peripheral cells of masses of *F. evenescens* eggs (again dividing the circle into two divisions of 180°) are as follows: in toward center of mass 260, out 8; in 38, out 0; in 113, out 3; in 210, out 2 etc. An example of a small isolated cluster of these eggs is shown in Fig. 2.

To answer the two questions raised in the first part of this paper, masses of closely-packed *F. vesiculosus* eggs were arranged in Syracuse dishes and in smaller dishes, and with a mouth pipette eggs of *F. evenescens* were placed at intervals around the periphery of each mass.

TABLE I

The results of seven experiments showing the directive effect of unfertilized *F. vesiculosus* eggs on the developing spores of *F. evenescens.*

Experiment No.	In	Out	Tangent	Equal Division
1	36	1	0	1
2	41	0	1	2
3	12	0	1	0
4	14	1	0	0
5	8	0	1	1
6	10	0	0	0
7	12	0	0	0
Totals	133	2	3	4

The *F. evenescens* eggs were placed at distances varying between one half and two egg diameters out from the periphery of the *F. vesiculosus* mass. Around the circumference the distance between consecutive *F. evenescens* eggs ranged from five to a hundred or more egg diameters, to rule out any effect which they might have on each other. (Other tests showed that no directive influence extended beyond four or five egg diameters.) To further control against any mutual effect of the *F. evenescens* eggs, in some cases the *F. evenescens* eggs were placed only on one side of the mass of *vesiculosus* eggs so that no other *evenescens* eggs whatsoever, at any distance, would be in the direction of the central *vesiculosus* mass. (Fig. 3.)

When newly shed and fertilized *F. evenescens* eggs were added in place, supernumerary *F. evenescens* sperm also came into the dish, but these did not fertilize the *F. vesiculosus* eggs of the central mass. The dishes were then placed in a dark room under black felt and were later examined without jarring the dishes. Since the fertilized spores adhere to the bottom of the dish they are not easily dislodged, but the unfertilized *vesiculosus* eggs are easily moved.

The results of seven experiments are given in Table I. In the column headed " in " are represented the eggs whose rhyzoids pointed inside an imaginary line tangent to the edge of the central mass. " Out " represents those which pointed outside the tangent line. " Tangent " represents the eggs whose rhyzoids paralleled the tangent line. " Equal " represents eggs which divided equally, without producing a rhyzoid. All four cases of equally dividing eggs developed cell plates parallel to the tangent line, as if to protrude rhyzoids directly inward (or out). The great preponderance of spores which divided with rhyzoids within the tangent line did so with the rhyzoid pointing almost exactly toward the nearest cell of the central mass. In experiments one, five, and six, the *F. evenescens* eggs were placed on only one side of the central mass.

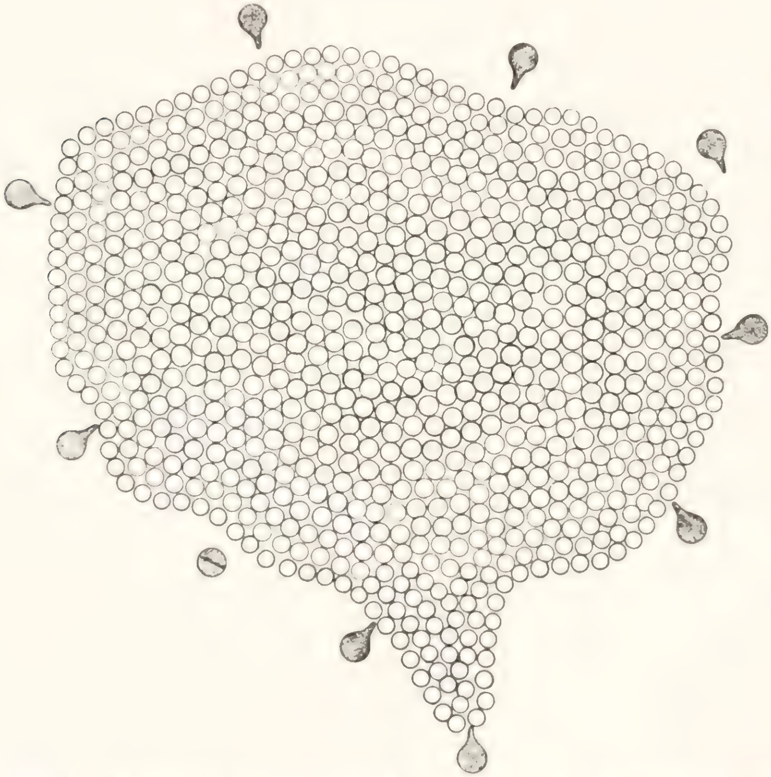

FIG. 4. The central mass consists of unfertilized resting *F. vesiculosus* eggs. At intervals around the periphery are directed *F. evenescens* spores, which are stippled. Their greater size has been slightly exaggerated. Reared in the dark.

In experiments two, three, four, and seven, they were placed all around the mass. The direction of the central mass from the *evenescens* eggs therefore covered all points of the compass and controlled against environmental asymmetries such as, for example, stray light (which, however, was not present). In experiments one and two the central mass was about one centimeter across; in experiments three, four, five, six, and seven, it was about two or three millimeters across, although the shape of the mass was not always strictly circular. A sketch of one of these experiments is given in Fig. 4. The peripheral *evenescens* cells are placed more closely together in this experiment than in the others.

Discussion of the Directive Agency in the "Group Effect"

The results show conclusively that eggs of *F. evenescens,* which tend to divide so that the rhyzoid grows in the resultant direction of neighboring cells, are equally well directed by unfertilized resting eggs of another species as by dividing eggs of the same species. The directive effect therefore cannot be ascribed to any agency dependent on nuclear or cell division in the directing cells. The effect is also non-specific, although the two species tested are of the same genus.

I am much indebted to Professor W. J. Crozier, in whose Laboratory these experiments were performed, for advice and criticism, and to Professor W. H. Weston and Dr. A. E. Navez for advice and help in locating the habitat of the *Fucus* and in the proper handling of the plants in order to obtain gametes. Professor W. R. Taylor has been so kind as to identify *F. evenescens* for me from a pressed specimen.

SUMMARY

1. The results of a number of observations and experiments are presented which relate to the nature of the *Fucus* egg and to some of the changes which take place in it at fertilization.

2. Giant eggs which in some cases develop supernumerary rhyzoids are found to originate in the fusion of single eggs within the capsule and especially at the time of emerging from the capsule.

3. The extent to which eggs fuse is found to be greatly reduced at low temperature and increased at high temperature.

4. Cross-fertilization between *F. vesiculosus* and *Ascophyllum nodosum* was found not to take place. The sperm of *F. evenescens* do not fertilize the eggs of *F. vesiculosus* to any appreciable extent. Individual receptacles of the monœcious *F. evenescens* are entirely self-fertile.

5. The developmental axis of the spores was found to be directed by the presence of nearby neighbors in *F. vesiculosus, F. evenescens,* and in *Ascophyllum.* The first division plane tends to lie so that the rhyzoid protrudes in the resultant direction of near neighbors. No jelly or other solid egg substance traverses the space between affected eggs.

6. Unfertilized resting eggs of another species were found to direct the division planes of eggs of *F. evenescens* equally well as dividing eggs of the same species. Therefore the directive effect cannot be ascribed to any agency dependent on nuclear or cell division in the directing cells.

BIBLIOGRAPHY

Farmer, J. B., and J. L. Williams, 1896. On Fertilization, and the Segmentation of the Spore, in *Fucus. Proc. Roy. Soc.,* **60:** 188.

Farmer, J. B., and J. L. Williams, 1898. Contributions to Our Knowledge of the Fucaceæ: Their Life-history and Cytology. *Phil. Trans. Roy. Soc. B.,* **190:** 623.

Hollaender, A., and E. Schoeffel, 1931. Mitogenetic Rays. *Quart. Rev. Biol.,* **6:** 215.

Hurd, Annie May, 1920. Effect of Unilateral Monochromatic Light and Group Orientation on the Polarity of Germinating *Fucus* Spores. *Bot. Gaz.,* **70:** 25.

Lund, E. J., 1923. Electrical Control of Organic Polarity in the Egg of *Fucus. Bot. Gaz.,* **76:** 288.

Morgan, T. H., 1924. Self-Fertility in Ciona in Relation to Cross-fertility. *Jour. Exper. Zoöl.,* **40:** 301.

Morgan, T. H., 1928. Experimental Embryology. Columbia University Press.

Pierce, G. J., 1906. Studies of Irritability in Plants. *Ann. Bot.,* **20:** 449.

Winkler, H., 1900. Ueber den Einfluss äusserer Factoren auf die Theilung der Eier von Cystosira barbata. *Ber. Deutsch. Bot. Gesells.,* **18:** 297.

Yamanouchi, Shigéo, 1909. Mitosis in *Fucus. Bot. Gaz.,* **47:** 173.

SPERMATOGENESIS IN THE CALIFORNIA OYSTER (OSTREA LURIDA)

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In connection with an investigation on the sequence of sexual phases in this species (Coe, 1931) numerous preparations were made of the gonads of oysters of definitely known ages. Some of these illustrate very clearly the general features of the processes concerned in the formation of the gametes. And since no very precise description of these processes has been published for any of the numerous species of oysters, it is hoped that this brief paper may be helpful in bringing to light some interesting deviations from the more usual types of spermatogenesis.

Nearly a half century ago Hoek (1883) published an excellent general account of the origin and growth of the gonads in *Ostrea edulis,* but he was unable to follow the cellular changes which occur in spermatogenesis. In the several papers by Orton, especially in the more recent extensive studies (1926) on the sex change in *O. edulis,* is much information on the characteristics of the gonads in each of the sexual phases. But these studies have not included gametogenesis.

It is well known that the spermatozoa of hermaphroditic species of oysters leave the body in the form of balls or ellipsoid clusters of closely packed ripe sperm cells. Each ball usually consists of from 250 to 2000 or more spermatozoa, each with its head directed toward the center of the ball and with its flagellum extending radially above the surface (Figs. 1, 4).

These balls are formed in the gonads and pass through the genital ducts into the mantle cavity, and thence out of the body, in great numbers whenever the animal in the ripe male phase is suitably stimulated.

In this connection it should be stated that all individuals of this species of oyster are protandric, and that there is a rhythmical alternation of female and male phases throughout the remainder of their lives (Coe, 1931). There are many intergrading stages in the change from one sex phase to the other, particularly in young animals. Furthermore, it frequently happens that one part of the reproductive system reaches a certain phase of sexuality in advance of other parts, whereby one portion of the system will have the characteristics of one sex while the rest of the gonads are predominantly of the other sex.

All grades of hermaphroditism are thus found in an oyster population at all seasons of the year. As a rule about half of the population may be roughly classed as intersexual forms, an equal number being predominantly of one sexual phase or the other. But animals exclusively male or female are few in number at any season, particularly those that are exclusively female.

The vast majority of those in the female phase either have more or less abundant sperm balls, remaining from the preceding male phase, in the follicles of the gonads or in the genital canals, or else they show some follicles in which the spermatogenesis for the succeeding male phase is anticipated. The ripe ova often mingle freely with the sperm balls in the genital ducts, but the firm attachment of each spermatozoön prevents self-fertilization, at least until after the sperm have been discharged into the mantle cavity.

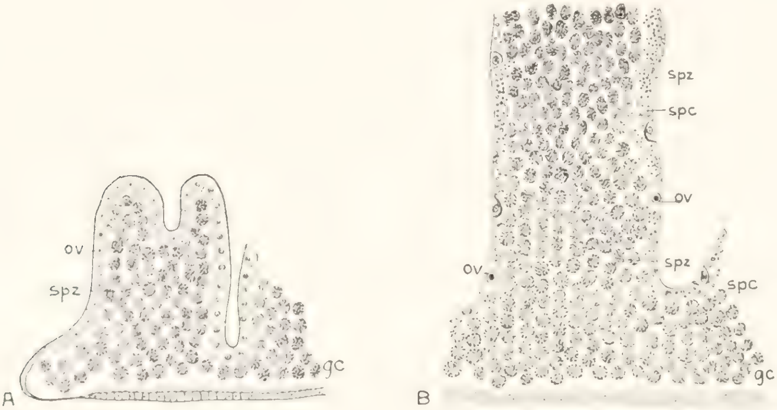

FIG. 1. Diagrams of portions of ripe gonads in first and third male phases drawn to the same scale. *A*, primary gonad, climax of first male phase, with ripe sperm-balls (*spz*) filling both lumen and ciliated genital canal (*gc*); follicle bordered with closely placed ovocytes (*ov*) anticipatory of first female phase. *B*, portion of secondary gonad, indicating its much greater size at climax of third male phase; very numerous balls (*spz*) now fill the much larger follicle and genital canal (*gc*); spermatocytes (*spc*) and a few large ovocytes (*ov*) border the lumen.

In the male phase similarly, particularly in young males, the follicles of the gonads usually contain more or less numerous ovogonia or well-grown ovocytes or both (Fig. 1). Only in the oldest animals are the transition stages almost eliminated.

In the young animal the first traces of the gonads appear at the age of about eight weeks. The few cells composing these gonads show no distinguishing characteristics of sexual differentiation, but at the age of twelve to sixteen weeks each gonad in every animal studied shows that both primitive ovogonia and spermatogonia are present. The spermatogonia, however, proliferate more rapidly than do the ovogonia and the gonad soon acquires the characteristics of a spermary although ovogonia and ovocytes are always present. Spermatogenesis quickly follows if the temperature is sufficiently warm and the ripe spermatozoa are ready to be discharged when the oyster is about five months of age (Fig. 2).

Before this initial male phase has been completed and before any of the sperm-balls have been discharged, the proliferation of the ovogonia and their transformation into ovocytes are in progress. Most of the sperm-balls are then discharged from the body, whereupon the animal assumes the first female phase, although some sperm-balls are always left in the genital ducts, and many spermatogonia for the subsequent male phase are present in the gonads.

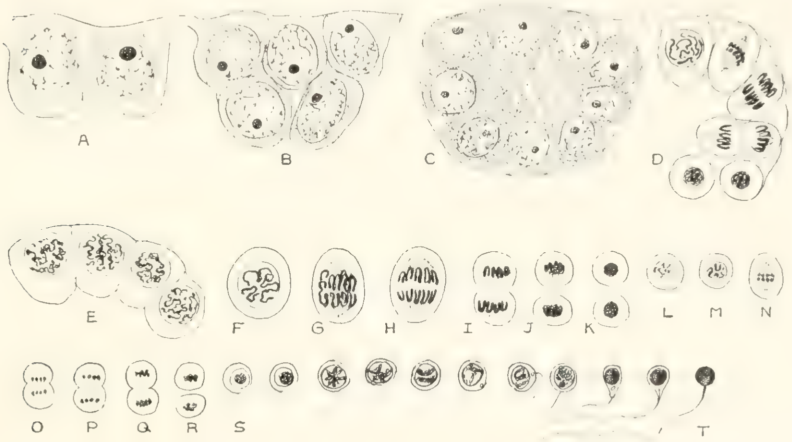

FIG. 2. Diagram of successive stages in spermatogenesis. *A,* two indifferent germ cells on wall of gonad; *B,* small group of spermatogonia, with reticular chromatin and conspicuous nucleoli; *C,* small group of secondary spermatogonia; *D,* division of secondary spermatogonia to form spermatocytes; *E,* primary spermatocytes with slender chromosomes; *F–K,* division of primary spermatocytes; *L–R,* division of secondary spermatocyte; *S, T,* transformation of spermatid into the mature spermatozoön.

In the female phase the ovocytes build up their yolk materials and ovulation occurs at the age of about six months. The eggs are retained in the mantle cavity of the parent during fertilization and cleavage and through development until the embryos have become provided with a bivalved, straight-hinged shell.

While the embryos are developing within the mantle cavity the spermatogonia remaining in the gonads begin a rapid spermatogenesis and even by the time the embryos have been spawned the second male phase has been reached. The number of sperm-balls produced is now vastly greater than in the first male phase and a much greater proportion of them contain the maximum number of spermatozoa. If the animal is well nourished some hundreds of thousands of such sperm masses are formed, with upwards of 2000 spermatozoa in each.

After the ripening and discharge of the sperm will come a recuperation period. And, apparently, these alternating sexual phases will be repeated regularly throughout the remainder of the animal's life. But it is not at all improbable that in certain individuals, and possibly in some hereditary strains, one sexual phase or the other may be considerably reduced in older animals, with a corresponding tendency toward a dioecious condition. Furthermore, if the nutritional conditions are favorable the recuperation period may be abbreviated or eliminated, resulting in several changes of the sexual phase in a single breeding season. Or a recuperation interval may divide any of the male phases, after the first, into two separate parts, one period of spermatogenesis immediately following ovulation and the other preceding the next female phase.

SPERMATOGENESIS

The successive stages in spermatogenesis will be discussed in the order in which they appear in the gonads of the young animal.

Indifferent gonia.—The earliest gonads, as found in young animals about eight weeks after attachment, consist of only a small number of cells and these show no recognizable characteristics that might indicate to which sex line they are ultimately destined. A few weeks later, however, after a large number of descendants has been produced, the two types of gonia are easily recognizable as such. The ovogonia then lie in a single row close beneath the surface of the gonad, while the primary spermatogonia occur singly or in small groups either against the wall or separated from it by several ovogonia (Figs. 1, 2).

Primary spermatogonia.—Each of the balls or clusters of ripe spermatozoa is derived from a single primary spermatogonium, and as there are commonly from 250 to 2000 or more spermatozoa in each ball the primary spermatogonium must divide six to nine times to produce the 64 to 500 or more secondary spermatogonia required. The number of divisions presumably depends upon the amount of nourishment available.

The first division of the primary spermatogonium is frequently vertical to the surface of the gonad, one of the two daughter cells remaining in contact with the surface (Fig. 2). Successive divisions result first in a morula of cells and then in a more or less regular spherical mass. Each of the constituent cells then assumes a pyramidal shape, with the apex toward the center of the group and with the nucleus near the base of the cell, that is, near the surface of the sphere (Figs. 2, 4). With still greater multiplication in numbers some of these secondary spermatogonia become crowded into the center of the sphere, causing a change in the shape of such as remain in contact with the surface. All the spermatogonia have large, vesicular nuclei, each with a conspicuous nucleolus and loose chromatin reticulum. Although the cell bodies are in close contact and are held together by a delicate non-cellular secretion, the cytoplasm of adjacent cells is always more or less completely separated (Fig. 2). In less well preserved specimens, however, the clusters may have the appearance of syncytial masses with nuclei imbedded radially in the common protoplasmic matrix.

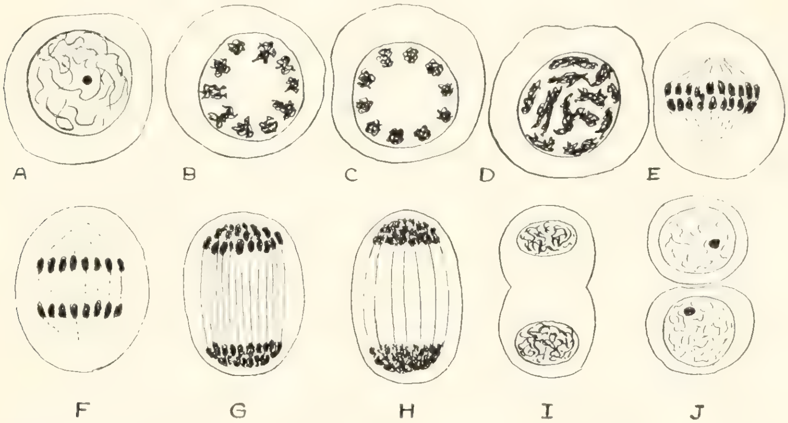

FIG. 3. Transformation and division of primary spermatocyte. *A*, leptotene; *B*, *C*, *D*, prophase groups of chromosomes; *E–J*, mitosis and formation of secondary spermatocytes.

Except at the time of mitosis the spermatogonia must absorb a considerable amount of nourishment, for the final cells remaining after the spermatogonial divisions have been completed are about one-eighth as large as was the original spermatogonium. The latter measures about .0057 mm. in diameter in the prepared sections while the final spermatogonia are about .0028 mm. in diameter. Intermediate gonia are intermediate in size.

Primary spermatocytes.—Following the last spermatogonial division the resulting primary spermatocytes are retained in the same spherical groups. Very little growth takes place, the nuclei soon showing the chromosomes in slender spiremes, apparently followed by the usual process of synapsis (Fig. 2). The typical brachytene stage soon appears and then the prochromosomes are arranged close beneath the nuclear membrane (Fig. 3). Prophase, metaphase, telophase are all of typical appearance, with a delicate spindle of the usual form. The chromosome number could not be definitely determined because of the crowded condition of the metaphase and anaphase plates, but it is not very large. There are two typical spermatocyte divisions.

Secondary spermatocytes.—The secondary spermatocytes are likewise held together in a crowded, spherical mass. Nuclear behavior and mitotic figures do not deviate from the typical condition (Figs. 2, 4).

Spermatids.—These also remain in close contact and become definitely oriented, each with its longer axis in a radial position in the irregularly spherical mass of from 250 to 2000 or more similar cells which compose the sperm-ball (Fig. 4).

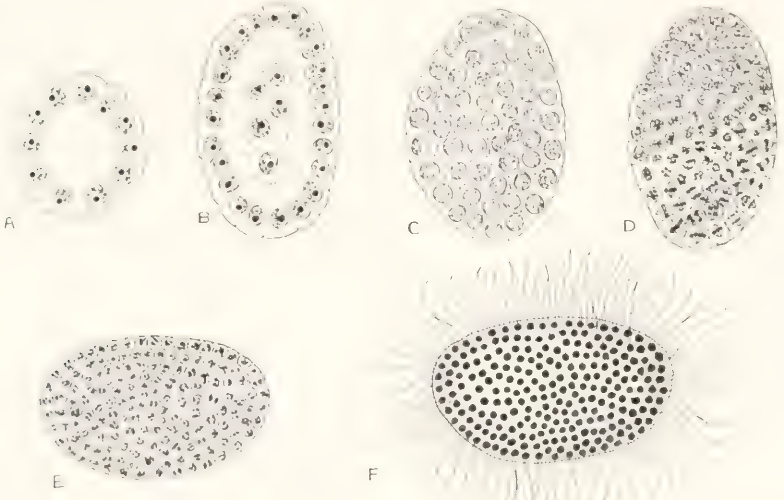

FIG. 4. Stages in formation of sperm-ball. *A*, group of young spermatogonia; *B*, later stage of same group; *C*, primary spermatocytes; *D*, division into secondary spermatocytes; *E*, spermatids; *F*, ripe sperm-ball, with radiating flagella of the spermatozoa.

Spermatozoa.—During the transformation of the spermatid to the spermatozoön the flagellum grows out radially and projects far beyond the surface of the group. The free outward growth of the flagellum shows that the sperm-ball has only a tenuous or gelatinous covering and that each spermatozoön is held in place by a common matrix of translucent gelatinous secretion.

Except for such relatively few groups of spermatogonia as have retained positions in contact with the wall of the gonad, the development of the sperm-balls has taken place in the fluid contained within the lumen. Usually the first sperm-balls to be completed are those adjacent to the spacious portion of the gonad which is continuous with the ciliated genital canals. Here they and the later ones accumulate until many thousands and in some cases perhaps hundreds of thousands are ready to be discharged (Fig. 1). The size of the balls varies greatly, due to variation in the number of the constituent spermatozoa, but most of them are between .04 and .06 mm. in longest diameter.

The male phase has now reached its climax and upon a suitable stimulus, such as a rise of temperature from below the critical point of 16° C. to above that point in the spring or, presumably, by the presence of eggs of other individuals in the vicinity at other times during the seven months of the breeding season, the spasmodic contractions of the oyster's body forces the myriads of sperm-balls into the water.

On reaching the sea water the sperm-balls rotate rapidly, due to the lashing of the tails of all the contained spermatozoa. The cementing substance is gradually dissolved, liberating the spermatozoa which are then free to swim about in the water. The ripe spermatozoön is not much more than .0012 mm. in diameter, with a flagellum about twenty times as long as the rounded oval head (Fig. 2). After all the spermatozoa have worked themselves free, the remainder of the intercellular matrix of the sperm-ball is left behind as an amorphous gelatinous material.

Comparison of the gonads of *O. virginica* with those of *O. lurida* shows that in both species there is a close agreement in the general features of spermatogenesis. But in the former species the derivatives both of the primary spermatogonia and of the spermatocytes separate freely, so that there is no aggregation into masses other than the association which results from the proximity of neighboring cells. The young spermatozoa are thus free to move individually in the lumens of the gonads and in the genital ducts, in marked contrast with those of *O. lurida,* where a special adaptation prevents, or diminishes the opportunities for, self-fertilization.

LITERATURE

COE, W. R., 1931. Sexual Rhythm in the California Oyster (*O. lurida*). *Science,* **74**: 247–249.

HOEK, P. P. C., 1883–84. De voortplantingsorganen van de oester: les organes de la génération de l'huitre. *Tijdrschr. Nederl. Dierkundige Ver.,* **1**: Suppl., 113–253.

ORTON, J. H., 1926–27. Observations and Experiments on Sex Change in the European Oyster (*O. edulis*). *Jour. Mar. Biol. Assn.,* **14**: 967–1045.

STUDIES OF PHOTODYNAMIC ACTION

III. The Difference in Mechanism Between Photodynamic Hemolysis and Hemolysis by Non-irradiated Eosine

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Dyes which bring about photodynamic hemolysis, in many instances, bring about the same effect in the absence of light (Dunkelwirkung), when the dye is present in sufficiently high concentration. This suggests the possibility that a reaction of the dye with cell constituents which is independent of light underlies the hemolysis; and that this reaction is accelerated by the activation of dye molecules by absorbed radiation, with the result that hemolysis occurs in lower concentrations of the dye. Certain rough correlations between the effect of the non-irradiated dyes and the photodynamic effects have been pointed out by Jodlbauer and Haffner (1921*a*) and by Blum (1930*b*) which would support this thesis, but it is possible that hemolysis is initiated by entirely different mechanisms in the two cases. Photodynamic hemolysis has been shown to require the presence of molecular oxygen (Hasselbalch, 1909; Schmidt and Norman, 1922), and there seems little doubt that this phenomenon is dependent upon oxidations by molecular oxygen activated in some way by light. Obviously, if the hemolysis produced by the non-irradiated dye is dependent upon the same reactions, it must likewise be inhibited by the absence of molecular oxygen. The attempt to separate the two processes on this basis has been the object of the following experiments.

Experimental

Quantitative experimental treatment of this problem meets with various difficulties. Measurement and comparison of the oxygen consumption during hemolysis by irradiated and non-irradiated dyes meets the *a priori* objection that certain dyes greatly alter the normal metabolism of cells without apparent destructive effects (see Barron and Hoffman, 1930), which might result in false conclusions as to the oxygen consumption of the process leading to hemolysis. Reducing the partial pressure of oxygen in any way, with the object of studying the effect on the hemolytic process, brings about changes in hydrogen ion concentration within the red blood cell due to the formation of reduced hemoglobin. This change in hydrogen ion concentration may considerably affect the hemolytic process (Jodlbauer and Haffner, 1921*b*; Blum, 1930*b*), without reference to oxidative reactions. Such factors offer considerable difficulties in quantitative experimentation, and it has, therefore, appeared wise to attempt only to demonstrate qualitatively, whether or not hemolysis by dyes may occur in the absence of light and oxygen.

The method employed has been as follows: Series of eosine solutions were prepared covering a range of concentrations which included the minimum concentration found to bring about hemolysis in air in the dark. The solutions were made up with isosmotic phosphate buffers usually at pH 7.0, according to the procedure described by Blum (1930*a*). Suspensions of 0.5 per cent red blood cells were made with these solutions, oxygen removed, and one series exposed to sunlight, while the other was maintained in the dark. If hemolysis by the non-irradiated dye, as well as photodynamic hemolysis, requires oxygen, hemolysis should not appear at any dye concentration in either the irradiated or the non-irradiated series. On the other hand, if the action of the non-irradiated dye does not require oxygen, there should be a concentration in each series above which hemolysis should occur. In the latter case the minimum concentration at which hemolysis occurs need not be exactly the same as the corresponding minimum in air, since the removal of oxygen would result in a change in the hydrogen ion concentration within the cell which might cause a shift in this minimum.

The validity of the results obtained in this way depends upon the removal of oxygen to a level which will not allow the oxidative mechanisms leading to hemolysis to proceed at a demonstrable rate. The only criterion for this is the complete inhibition of hemolysis in the irradiated systems. This was found difficult, the difficulty lying apparently in the removal of the oxygen from the cells themselves. The suspensions which we have found convenient for the study of the hemolytic process contain 0.5 per cent red blood cells, and it may be readily calculated that in such a system the cells contain about one-fourth of the total oxygen in the system. The cells contain about forty per cent by volume of oxygen, or 0.2 cc. per 100 cc. of suspension containing 0.5 per cent cells. From the absorption coefficient of oxygen in water approximately three cc. of oxygen are absorbed in 100 cc. of water at 25° C., and since our solutions are saturated with air and not with oxygen, they should contain approximately one-fifth of this quantity or 0.6 cc. per 100 cubic centimeters. Thus there is, roughly, one-third as much loosely-bound oxygen in the cells as there is dissolved oxygen in the surrounding solution. It is thus apparent that the oxygen must be removed from the cell as well as from the solution in order to establish the desired low oxygen tension. It was not found possible to completely inhibit the effects of the irradiated dye by reducing the atmospheric pressure above the solution. It was likewise found difficult to obtain conclusive results by attempting to remove the oxygen by bubbling nitrogen through the solutions for a considerable time. It was found, however, that definite results could be obtained by using carbon monoxide to remove the oxygen from the cells. The procedure in these cases was first to bubble nitrogen through a series of tubes, each containing 2 cc. of dye solution of a given concentration without cells for 15 to 20 minutes to ensure the removal of oxygen from the solutions. Carbon monoxide was bubbled through a 50 per cent suspension of washed red blood cells to remove the oxygen from these; 0.2 cc. of this suspension was then added to each tube to form a 0.5 per cent suspension of cells, the tubes being opened to the air for as short a time as possible in order to avoid the entrance of oxygen. The suspensions were then flushed out with about 400 cc. of carbon monoxide. Such treatment was found very effective in inhibiting the photo-reaction, but, as will be seen by reference to Table I, did not inhibit the action of the non-irradiated dye. In order to rule out any possible specific effect of carbon monoxide, similar systems were treated with a mixture of 20 per cent oxygen and 80 per cent carbon monoxide.

TABLE I

Irradiated systems exposed to mid-day sunlight for 1 hour (12:15 p.m.–1:15 p.m. August 21, 1931). Observations made at the end of 6 hours following mixing of cells with dye solution. *H* = complete hemolysis; *(H)* = partial hemolysis. Solutions contain sodium phosphate buffer isosmotic with 0.15 M NaCl, pH 7.0, + 0.5 per cent r. b. c. Human.

Eosin Concentration	*a.* Systems in Air		*b.* Systems in CO		*c.* Systems in $\frac{CO}{O_2} = \frac{80}{20}$	
	Irradiated	Not Irradiated	Irradiated	Not Irradiated	Irradiated	Not Irradiated
per cent						
1.4	H	H	H	(H)	H	H
.7	H	(H)	H	(H)	H	(H)
.35	(H)	—	—	—	(H)	—
.175	(H)	—	—	—	(H)	—
.087	(H)	—	—	—	(H)	—
.044	H	—	—	—	H	—

Table I presents the results of a typical experiment, in which three series of cell suspensions were exposed to sunlight, (*a*) in air, (*b*) in carbon monoxide, and (*c*) in a mixture of carbon monoxide and oxygen, while three similar series were maintained in the dark. The results show that the photodynamic effects are completely inhibited by the absence of oxygen, while the effects of the non-irradiated dye are not. The fact that the photo-effect is completely inhibited in an atmosphere of carbon monoxide indicates that the oxygen content of the system has been lowered sufficiently so that hemolysis by the non-irradiated dye should also be inhibited if it is dependent upon the same oxidative reactions as the photodynamic effect. The fact that carbon monoxide does not inhibit the light reaction when oxygen is present indicates that the inhibition is not a specific action of the carbon monoxide but is due to lack of oxygen. While the results of such experiments vary somewhat with regard to the rate of development of hemolysis, in no case has it been possible to completely inhibit hemolysis by non-irradiated dyes. A certain amount of the variation may be due to temperature differences. The non-irradiated systems were maintained at a temperature of approximately 25° C. during the period before the observations were made. On the other hand, the irradiated systems were exposed during the one-hour period of the irradiation to a variable temperature, which, however, was never higher than 27° C. and in some experiments was considerably lower than the temperature of the non-irradiated systems. So far as can be determined, by such qualitative observation as we have used, the rate of hemolysis is somewhat decreased in the systems in contact with CO or mixtures of CO and O_2. It seems probable that this is due to the difference in hydrogen ion concentration of the cells containing carboxyhemoglobin from that of those containing oxyhemoglobin. It is possible, of course, that in the series exposed to light, the carboxyhemoglobin is dissociated to some extent by the action of light with the formation of oxyhemoglobin or reduced hemoglobin, depending upon whether oxygen is present or not. This might account for some differences in the rate of hemolysis between irradiated and non-irradiated systems.

The fact that hemolysis is completely inhibited in the absence of oxygen excludes the possibility that reactions of the type described by Levaillant (1923) and Windaus and Borgeaud (1928) may bring about the destructive changes leading to hemolysis. These reactions take place in the absence of oxygen and may be considered as oxidations in which the dye acts as a hydrogen acceptor. The dye is reduced in these cases to the colorless leucobase, and the fact that no bleaching of the dye was observed in our experiments indicates that reactions of this type did not occur to any appreciable extent.

While it was not found possible, as mentioned above, to inhibit the photo-effect completely by evacuation or by bubbling nitrogen through the suspensions, it was found that such treatment markedly decreased the photo-effect, but did not alter the hemolytic effect of the non-irradiated dye. Similar results were observed when the attempt was made to inhibit these reactions by the use of reducing agents in the solution. Experiments were carried out using Na_2SO_3, $Na_2S_2O_3$, and $NaNO_3$ in concentrations up to 0.1M. It was found impossible to completely inhibit the photo-reaction by means of these reducing agents, and therefore it cannot be assumed that the reducing power of the solution is sufficient to completely inhibit the action of the non-irradiated dye if it involves the same process as the photo-reaction. However, the fact that these reagents have no apparent tendency to inhibit the dark reaction indicates that the two processes are essentially different. Thus, while the above evidence may not, perhaps, be considered as absolutely conclusive, collectively it gives strong support to the view that the dark reaction is not an oxidation by molecular oxygen, whereas the photo-reaction is.

Discussion

Hasselbalch (1909) performed experiments to test whether the hemolysis by non-irradiated dyes requires oxygen, and reported results contrary to those described above. Using red blood cells in suspension in isotonic NaCl solution which contained a given concentration of dye sufficient to bring about hemolysis in the absence of light, he found that the evacuation of the air above the solutions inhibited the hemolysis. Evacuation under these conditions would remove CO_2 as well as O_2, and since the solutions in which the cells were suspended were unbuffered, the removal of CO_2 must have decreased their hydrogen ion concentration. Furthermore, the removal of both CO_2 and O_2 from the cells themselves must have resulted in a decrease of hydrogen ion concentration within the cells due to formation of reduced hemoglobin. Hemolysis by non-irradiated fluorescein dyes is markedly affected by hydrogen ion concentration (Jodlbauer and Haffner, 1921*b*; Blum, 1930*a*), the minimum concentration necessary to bring about hemolysis increasing as the hydrogen ion concentration decreases. Thus it seems quite probable that the results obtained by Hasselbalch with fluorescein dyes (eosine and rose bengal) were due to the decrease of hydrogen ion concentration to a value at which the concentration of the dye employed would not produce hemolysis in the absence of light. The justification of this criticism will appear upon the examination of the tables given by Blum (1930*b*).[1] Hasselbalch also used quinine hydrochloride and quinine bisulphate, finding that evacuation prevented hemolysis in the former but not in the latter case. This variation in effect indicates that the factor affecting hemolysis was probably something other than the O_2 content of the system. Although, as stated above, changes of hydrogen ion concentration must have occurred in our systems due to the formation of carboxyhemoglobin, this did not mask the effect of O_2 lack because the observations were made over a wide range of dye concentrations.

Experimental—The Effect of Cyanide on Photodynamic Hemolysis

It has been suggested that the mechanism of photodynamic action involves the normal respiratory mechanisms of the cells themselves (*e.g.*, Metzner, 1919 and 1921). If this were true, photodynamic hemolysis should be inhibited by the inhibition of the respiratory enzymes. In the above experiments it was found that the photodynamic effects are not inhibited by CO provided O_2 is present. The presence of CO should partially inhibit all the respiratory oxidative mechanisms of the cell except the aerobic dehydrases.[2] However, light decreases the inhibitory effect of CO on certain of these mechanisms ("respiratory enzyme" of Warburg, "indophenol oxidase" of Keilin), and it is possible that the inhibitory effect of CO was very slight in the systems where mixtures of CO and O_2 were used (Warburg, 1926).

To test this question further, cyanide was used to inhibit respiratory enzymes. Series of dilutions of eosine were prepared as above, to a part of which M/100 KCN was added. Red blood cells were added (0.5 per cent) and a part of the KCN series was exposed to light together with control series not containing KCN; other KCN and control series were maintained in the dark. In no case could a difference be detected between the KCN series and the controls in either the irradiated or the non-irradiated systems. These experiments are in agreement with those of Loeb (1907) and Moore (1928), who found that KCN did not inhibit destructive changes in echinoderm eggs by eosine and sunlight, and of Baumberger et al. (1929), who found that cyanide did not inhibit the photodynamic action of methylene blue in preventing the clotting of blood plasma. Cooke and Loeb (1909) found that KCN increased the photodynamic effects of some dyes on eggs, but this may have been due to hydrogen ion concentration effects.

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[1] The values for molar concentrations of dye given in these tables are in error; the decimal point should in all cases be moved one place to the right. Hasselbalch used M/200 eosine in his experiments, and it will be seen that this concentration is, according to these tables, one at which a small difference in hydrogen ion concentration might determine the occurrence or non-occurrence of hemolysis.

[2] The nomenclature here used is that of Dixon (1929).

The addition of M/100 KCN should serve to inhibit markedly all the known respiratory mechanisms with the exception of the aërobic dehydrases, including those in which light interferes with inhibition by carbon monoxide. If these mechanisms played a part in the production of photodynamic hemolysis, the inhibitory effect of the cyanide should be reflected in a reduction of hemolysis. As stated above, no such decrease could be observed.

While it is possible that the aërobic dehydrases may play a part, it seems probable that the photodynamic effects are the result of direct oxidation of cell constituents by molecular oxygen, the activation of the O_2 resulting from light energy absorbed by a sensitizer and completely independent of activation by cellular enzymes. The destruction of respiratory enzymes might play a more important part in cells in which respiration is more active than in red blood cells, and may possibly account for the induced tropisms of Metzner (1919, 1921), as he suggests, but this explanation has no experimental support.

These experiments also suggest that hydrogen peroxide is not formed as an intermediate step in photodynamic action. If H_2O_2 took a part in the oxidations, catalase should tend to oppose the photodynamic effect by its destruction; in such a case the inhibition of catalase by cyanide should result in increased photodynamic effects. As stated above, cyanide has no effect whatsoever on photodynamic hemolysis, and since this is true, it appears improbable that H_2O_2 is formed as an intermediate. This does not, however, deny the formation of intermediate organic peroxides which would not be attacked by catalase.

SUMMARY

1. The absence of molecular oxygen completely inhibits photodynamic hemolysis but does not inhibit the hemolytic action of the non-irradiated dye. The two phenomena are thus dependent upon different fundamental mechanisms.

2. Cyanide does not inhibit hemolysis either by the irradiated or non-irradiated dye. Thus the respiratory mechanisms of the cell, with the exception of the aërobic dehydrases, cannot play a part in the production of photodynamic hemolysis.

BIBLIOGRAPHY

Barron, E. S. G., and L. A. Hoffman, 1930. The Catalytic Effect of Dyes on the Oxygen Consumption of Living Cells. *Jour. Gen. Physiol.,* **13:** 483.

Baumberger, J. P., R. T. Bigotti, and K. Bardwell, 1929. The Photodynamic Action of Methylene Blue on the Clotting Process. *Proc. XIIIth Int. Physiol. Cong., Am. Jour. Physiol.,* **90:** 277.

Blum, H. F., 1930*a*. Studies of Photodynamic Action. I. Hemolysis by previously irradiated fluorescein dyes. *Biol. Bull.,* **58:** 224.

Blum, H. F., 1930*b*. Studies of Photodynamic Action. II. The relationship between hemolysis by irradiated and non-irradiated eosine. *Biol. Bull.,* **59:** 81.

Cooke, E., and L. Loeb, 1909. Über die Giftigkeit einiger Farbstoffe für die Eier von Asterias und von Fundulus. *Biochem. Zeitschr.,* **20:** 167.

Dixon, M., 1929. Oxidation Mechanisms in Animal Tissues. *Biol. Rev.,* **4:** 352.

Hasselbalch, K. A., 1909. Untersuchungen über die Wirkung des Lichtes auf Blutfarbstoffe und rote Blutkörperchen wie auch über optische Sensibilisation für diese Lichtwirkungen. *Biochem. Zeitschr.,* **19:** 435.

Jodlbauer, A., and F. Haffner, 1921*a*. Über den Zussamenhang von Dunkelwirkung fluorescierender Stoffe und Photodynamie auf Zellen. *Biochem. Zeitschr.,* **118:** 150.

Jodlbauer, A., and F. Haffner, 1921*b*. Über die Wirkung von Eosin und Rose bengale auf rote Blutkörperchen und den Zussamenhang von Aufnahme und biologischer Wirkung. *Pflüger's Arch.,* **189:** 243.

Levaillant, R., 1923. Fluorescence et Photochimie. *Compt. Rend. Acad. Sci.,* **177:** 398.

Loeb, L., 1907. Über den Einfluss des Lichtes auf die Färbung und die Entwicklung von Eiern von Asterias in Lösungen verchiedener Farbstoffe. *Arch. f. Entwick.-mech.,* **23:** 359.

Metzner, P., 1919. Über die Wirkung photodynamischer Stoffe auf Spirillum volutans und die Beziehungen der photodynamischen Erscheinung zur Phototaxis. *Biochem. Zeitschr.,* **101:** 33.

Metzner, P., 1921. Zur Kenntnis der photodynamischen Erscheinung: Die induzierte Phototaxis bei Paramecium caudatum. *Biochem. Zeitschr.,* **113:** 145.

Moore, A. R., 1928. Photodynamic Effects of Eosin on the Eggs of the Sea Urchin, Strongylocentrotus purpuratus. *Arch. di Sci. Biol.,* **12:** 231.

Schmidt, C. L. A., and G. F. Norman, 1922. Further Studies on Eosin Hemolysis. *Jour. Gen. Physiol.,* **4:** 681.

Warburg, O., 1926. Über die Wirkung des Kohlenoxyds auf den Stoffwechsel der Hefe. *Biochem. Zeitschr.,* **177:** 471.

Windaus, A., and P. Borgeaud, 1928. Über die photochemische Dehydrierung des Ergosterins. *Liebig's Ann.,* **460:** 235.

THE MATURATION DIVISIONS AND SEGREGATION OF HETEROMORPHIC HOMOLOGOUS CHROMOSOMES IN ACRIDIDAE (ORTHOPTERA)

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Contents

I. Introduction 324
  1. Definition of a chromosome 325
  2. Time of segregation 325
  3. The historically correct usage of the term maturation as applied to gametogenesis 329
II. Material and acknowledgments 331
III. Observations 332
  1. Segregation of unequal homologues 332
    (1) *Trimerotropis citrina* 332
    (2) *Mecostethus gracilis* 338
    (3) *Amphitornus bicolor* 338
  2. Chromomere vesicles and the origin of unequal homologues 339
  3. An accident during mitosis, *A. bicolor* 341
  4. Incipient octad formation, *T. citrina* 341
IV. Discussion 347
V. Literature list 348
VI. Explanation of plates
VII. Plates

I. Introduction

The presentation of scientific facts to students and to the public requires coördinated efforts between research workers on the one hand and authors of text-books and popular articles on the other. Certainly, the duty of an investigator, when a group of facts sufficient to justify a conclusion has been definitely ascertained, is to present both the facts and the conclusion in a clear and concise form through the proper channels. But the obligation of one who prepares a text-book is no less to keep diligently in touch with these sources of information and to present adequately and accurately the various subjects with which he deals.

The whole field of biology presents no more clear, simple and beautifully logical process than that of maturation. Yet, judging from the presentation of this important subject in current text-books and the conceptions derived from these presentations by university students, someone has failed in his duty. Serious consideration of the problem of responsibility leads me to the conclusion that the blame lies largely with the cytologists. The facts necessary to a clear comprehension of the mechanism of maturation have been available for a number of years though certainly not in a particularly clear or readily accessible form, when one considers the amount of attention the author of a general text-book can give to the subject.

The difficulties seem to be due chiefly to hazy conceptions concerning three points: (1) Definition of a chromosome; (2) time of segregation and (3) the historically correct usage of the term maturation as applied to gametogenesis. These will be considered in turn.

1. *Definition of a Chromosome.*—Little excuse exists for the misuse of this word. Waldeyer (1888) named and defined chromosomes as the individual, rod or loop-shaped, longitudinally split, basophilic bodies which are formed from the nuclear network during mitosis. McClung (1905) amplified this definition as follows: "Chromosomes are chromatic elements acting as unit structures during mitosis. Chromosomes are of two general classes:

1. Simple—containing two chromatids in metaphase.
2. Multiple—containing more than two chromatids in metaphase and formed by the union of simple chromosomes.
    - (*a*) Tetrads, containing four chromatids (derived from a pair of homologues, as ordinarily used.)
    - (*b*) Hexads, containing six chromatids.
    - (*c*) Octads, containing eight chromatids (etc.)."

"A chromatid is a (longitudinal) half of a simple chromosome." The parts in parenthesis are mine. In other words, chromosomes are the individual, chromatic elements which appear definitely in the nucleus at the end of the prophase and which act as unit structures during mitosis.

2. *Time of Segregation.*—There is a very wide-spread habit among biologists, especially geneticists, of referring to one of the maturation divisions as the reduction and the other as the equation division. The facts in regard to the segregation of the sex-chromosomes in a number of organisms have been available for years, and they stand in direct contradiction to such views. The true status may be seen from the following data summarized from the recent edition of E. B. Wilson's valuable book:

	Accessory		XY	
	Pre-reduction	Post-reduction	Pre-reduction	Post-reduction
Orthoptera	10 genera		1 genus	
Homoptera	4 genera			
Heteroptera	5 genera	4 genera		11 genera
Coleoptera	2 genera	2 genera	6 genera	
Nematoda	2 genera	2 genera	1 genus	
Diptera			3 genera	

Other evidence, which has been available for years, demonstrates that at least some of the euchromosomes behave in a similar manner. Wenrich (1916) showed that the homologues of the tetrad which he designated as C_1 segregate half the time in the first and half the time in second maturation division. He also showed that the homologues of tetrad B and other combinations of tetrad C segregate uniformly in the second division, while the writer (1913) and Robertson (1916) had shown that segregation, in what are probably comparable tetrads of certain other species, occurs constantly at the first division. Obviously, therefore, neither division can be referred to accurately as *the* segregation (reduction) division; the term is applicable only to the separation of the maternal and paternal components of any chromosome and not to either maturation division. Further evidence that the object to be attained and not the time of attaining it is the essential feature of maturation will be presented in this paper.

Quotations from a few of the best current text-books of biology will illustrate these points. The following statement occurs in one à propos of the first maturation division:

> "But, and this is the crucial point, in the early anaphase the members of each pair are separated, one synaptic mate going to each pole of the spindle. Thus each of the daughter cells—SECONDARY SPERMATOCYTES—receives half the total number of chromosomes that were present in the primary spermatocyte or the somatic cells. The essential difference between this type of mitosis (REDUCTIONAL DIVISION) and that involved in other nuclear divisions (EQUATION DIVISIONS) lies in the separation of entire chromosomes (synaptic mates) instead of the splitting of each chromosome."

This book is entitled "Foundations of Biology" and the author states in the preface that it is intended for college students and the general reader. Yet, among the first things that a college student in many laboratory courses in zoölogy will see for himself, is the fact that the *number* of chromosomes in the first oöcyte or spermatocyte is one-half that in the somatic cells and is the same as the number in the second oöcyte or spermatocyte. Such a statement, therefore, confuses the student or else gives him an incorrect conception as to the use of the word chromosome. Furthermore, it gives him an erroneous idea as to the process and time of segregation, which as already mentioned is not confined to either maturation division, exclusively.

The following quotation from another author shows improvement over the preceding in that there is recognition of the fact that segregation may occur at either division, but a similar lack of a clear conception of a chromosome:

> "*Tetrads.*—The pairs of chromosomes often do not appear as double bodies; for while the chromosomes have been coming together they may also have divided. Each pair thus consists of four half-chromosomes; and the quadruple body formed is called a *tetrad.* Owing to its origin, two of the parts of each tetrad are maternal, the other two paternal. In the two maturation divisions the tetrads are divided, in two planes, first into double bodies called *dyads*, next into their single components."
>
> "*First Maturation Division.*—A spindle is formed, on which the tetrads take their place. How the tetrads are divided depends on the way they are placed on the spindle. In part, this position appears to be fixed and always the same in the same species. In the illustration they are represented as having been so placed that the maternal half of the tetrad is separated from the paternal half. It is a matter of chance, however, whether the paternal half is turned toward one end of the spindle or toward the other. It may happen therefore, that all of the paternal dyads go into one cell and all of the maternal dyads into the other, or, as in the figure, part into one cell and part into the other. The cells produced by this division are called *secondary spermatocytes.*"
>
> "It is worthy of note that in the division just described, no chromosomes have divided. The tetrads have divided, but merely by the separation of the two chromosomes which had previously come together. Such a division is called a *reductive division;* it never occurs in cell divisions except in maturation, and in only one of the maturation divisions."

Tetrads are chromosomes according to any accepted definition of the word; hence, "chromosomes" *have* divided in the above instance. Also since a tetrad is a chromosome the statement that it is four "half-chromosomes" is rather confusing. The difficulty disappears if the statement is changed to "Each pair thus consists of four chromatids." Two other points in the above quotation, while actually accurate, are inadequate, so far as the inexperienced student is concerned. One is: "It may happen, therefore, that all of the paternal dyads go into one cell and all of the maternal dyads into the other." The student gains the idea that this is a reasonably frequent occurrence, whereas, in an organism like a short-horned grasshopper with its 24 or 23 chromosomes (12 pairs), assuming the simplest possible conditions with no crossing-over, one gamete in each 4096 would be expected to contain the haploid set of 12 chromosomes contributed by a particular parent (2^{12}): while, in man with 48 chromosomes (24 pairs), the ratio is 1:16-777216 (2^{24}). The other misleading statement concerns the reduction division: ". . . it never occurs in cell divisions except in maturation and in only one of the maturation divisions." As a matter of fact, as previously pointed out, reduction (better, segregation) occurs in both maturation divisions; but, obviously, a given pair can segregate in only one, though a corresponding pair in another cell may segregate in the other. In other words, the term reduction division should only refer to the separation of the maternal and paternal components of any chromosome and not to either maturation division taken as a whole.

May I present just one more quotation on this subject from another author:

> ". . . Weismann in 1888 prophesied that in one of the maturation divisions it would be found that the chromosomes do not divide longitudinally but transversely so that the hereditary characteristics instead of being equally partitioned between the daughter cells would be divided crosswise so that the daughter cells would receive dissimilar groups of biophors. The ordinary longitudinal division of the chromosomes he called an *equation division* and the extraordinary hypothetical division during maturation the *reduction division*."
>
> "The fulfilment of this prophecy by a host of different observers was a remarkable justification of the imagination in science. The reduction division in some form or other, often complicated and atypical, was revealed in type after type of animals and plants until today it is generally if not quite universally accepted as a typical phenomenon of maturation."

Of course, the last sentence may be stretched to cover a multitude of views, but the idea which is conveyed to the student as one to be accepted without question is that there is a transverse or cross division of chromosomes. Weismann realized that reduction in number of "ids" must occur before fertilization; otherwise, the number would be doubled each generation. He predicted that reduction would be found to occur during maturation and suggested two ways in which it might be brought about, either by a sorting out of chromosomes into two similar groups, one of which would go to each pole without division of the constituent idants (chromosomes) or by a transverse instead of a longitudinal division of each individual chromosome. For the intrinsic process, whatever the method, he proposed the term *reduction division*. The ordinary longitudinal division was already known as an *equation division*.

The cytologists of the time were quick to show that no transverse division of the chromosomes occurs and that Weismann's first suggestion was the correct one. The mechanism which insures the separation of the members of the two groups is the initial reduction in number of chromosomes through the synapsis of longitudinally split homologues (pseudo-reduction) followed by their actual distribution to different cells by the two following divisions. The division which separates the parts of any chromosome derived from one parent from those derived from the other is the reduction division in Weismann's sense for that particular pair of homologues. So much will do for the first two points, the last remains to be considered.

3. *The Historically Correct Usage of the Word Maturation as Applied to Gametogenesis.*—The early usage of the terms spermatogenesis, oögenesis and maturation was perfectly logical and clear-cut. One group of investigators was concerned with the origin and early history of the germ cells and called the entire process spermatogenesis in the male and oögenesis in the female. The other group was interested in the ripening (maturation) of the egg and its attendant phenomena.

A brief survey of the two groups will make my point clearer. In the first, we find v. la Valette St. George who from 1865–76 published a series of four papers entitled, "Ueber die Genese der Samenkörper." [1] Much of our present terminology on spermatogenesis was proposed by St. George in the last of these papers. In this group fall, also, the following men who were largely instrumental in developing the theory of the continuity of the germplasm; Richard Owen, 1849, called attention to certain distinctions between body and germ cells. Virchow, 1858, was led to enunciate his famous dictum, "Omnis cellula e cellula." Certainly, recognition of the fact that cells arise only from preëxisting cells was an essential step in establishing the idea of the continuity of the germplasm. Jaeger, 1878, used the expression "Continuitat des Keimprotoplasma." Credit for establishing this theory, however, goes to Nussbaum whose work, 1880, on the early development of the frog and trout led him to a clear statement of the concept of the continuity of the germ cells and of the evidence for his conclusions. And finally, Weismann, 1883, directed attention to the bearing of the continuity and comparative isolation of the germ cells on theories of evolution and heredity.

Leaving this hasty summary of the early work on spermatogenesis and oögenesis, let us turn to the other group of investigators; who were concerned with neither the origin nor the early development of the germ cells but with the maturation (ripening)of the egg. (As we shall see, the sperm was not supposed to undergo such a process until Van Beneden recognized the essential feature of polar body formation to be the elimination of chromatin.)

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[1] This was not the end of the series.

The gradual recognition of the essential features of the ripening of the egg may be summarized briefly as follows: The germinal vesicle was discovered by Purkinje, 1825. A polar body was first figured, apparently, by Carus, 1824, for a mollusk egg. Carus, however, gave no adequate description of the structure and offered no suggestion as to its function. Von Baer, 1827, noted in the hen's egg the migration of the germinal vesicle to the periphery of the yolk and its disappearance. He believed both processes to be concerned with the maturation of the egg. Dumortier, 1837, saw and described the two polar bodies in a mollusk egg. He believed them to be the Purkinje (germinal) vesicle. F. Müller, 1848, suggested that these bodies were concerned in the determination of the early cleavage planes and accordingly applied the name "Richtungsbläschen" to them. Robin, 1862, in recognition of this constant relation to the cleavage planes called them "Globules polaires," hence our term polar bodies. Mark, 1881, was the first to suggest that the polar bodies should be regarded as rudimentary eggs. Somewhat earlier, 1875, Van Beneden wrote that maturation clearly consisted in the breaking down of the germinal vesicle, the formation of the polocytes and the return of the nucleus into the yolk. By 1883 the same author had worked out the relation of the chromatin to polar body formation and had recognized this as the essential feature of the ripening of the egg. He also noted the equivalence of the male and female pronuclei in regard to amount of chromatin. (The word chromosome was not coined until 1888.) He then prophesied that a process whereby the amount of chromatin is reduced would be found to occur in spermatogenesis, and later, 1887, in collaboration with Julin demonstrated such to be the case, and that the sperm as well as the egg underwent maturation, the essential feature of which is chromatin reduction. Only in later text-books do we find such a confusion of ideas as may be illustrated by this quotation: "The maturation of germ cells in the male is called spermatogenesis, in the female oögenesis." Let me repeat that in contrast with the idea of maturation as a process concerned with the reduction in amount of chromatin the concept of gametogenesis includes the entire process from the time the germ cells are first recognized through multiplication, growth, maturation, and in the male, transformation of the sperm.

This may seem to be an unusual introduction to a scientific paper, but I can only add that in dealing with advanced students in Zoölogy

I have found their instruction faulty on the above points and believe that the confusion is not necessary. In any case, the present paper is concerned chiefly with the second of these points, the time of segregation. The other two are matters of definition and priority of usage.

The problem, then, is: When does segregation (reduction in Weismann's sense) occur? As already shown, this question can apply only to individual pairs of homologues and not to either maturation division. Three conditions render the answer difficult. First, usually the homologous chromosomes are indistinguishable morphologically. Second, parasynapsis is the method of union, at least in many forms, and is preceded by the splitting of the homologues. Finally, the four chromatids which are parallel during part of the prophase, later form equal-armed crosses and figures of 8 in such a manner that the chromatids which are together in one arm or loop are separated in the next.

Wilson, McClung and Wenrich are among the few who, when convinced of parasynapsis, recognized at once that, in view of the structure of the later prophase figures, they could not determine which of the four chromatids came from a given parent, and hence, which division separated sister chromatids and which homologues.

We must resort then to other means for determining when segregation occurs. Information is available from four sources. (1) Sex chromosomes, either XY pairs or the unpaired accessory chromosome; (2) Heteromorphic homologous chromosomes; (3) Polyploidy, and (4) Genetical evidence. The data from the first have been summarized, the last two will be considered in the discussion, while evidence to be presented concerns the second. When the homologues are unlike in size or shape it is a simple matter to observe when segregation occurs.

II. Material and Acknowledgments

This detailed study of segregation of unequal homologues is based on males from one species, each, of three genera of short-horned grasshoppers, distributed as follows: 71 *Trimerotropis citrina* from Kansas, Texas and Florida, 10 *Mecostethus gracilis* from Maine and Michigan and a number of *Amphitornis bicolor* from Kansas and Colorado. In each of these three species certain individuals have one or both of the two smallest pairs of chromosomes composed of homologues of different sizes.

I am indebted to Dr. W. R. B. Robertson for *T. citrina* from Lawrence, Kansas and to Dr. H. B. Baker for *M. gracilis* from the University of Michigan Biological Station near Cheboygan, Michigan. The work was done at the Marine Biological Station at Woods Hole and the Zoölogical Laboratory of the University of Pennsylvania.

III. OBSERVATIONS

1. *Segregation of Unequal Homologues.*—(1) *Trimerotropis citrina:*[2] A spermatogonial complex from a typical individual of this species consists of 23 telomitic chromosomes which may be arranged according to size in two groups. The first is composed of two small pairs. The members of one of these pairs are about two-thirds the size of those of the other pair. The second group consists of nine closely graded pairs and the accessory. There is a decided break between the two groups; as shown in Plate I, Fig. 1, the members of the smallest pair in the second group are more than twice the length of the members of the larger pair of the first group. In this and the following plate, the members of the two smallest pairs are shown in solid black in order to facilitate recognition. Figure 4 is a side view of a first spermatocyte from an individual with a spermatogonial complex such as has just been described, while Fig. 11 is a polar view of a second spermatocyte with a corresponding complex.

Sixty of the seventy-one specimens studied are, chromosomally, of the sort just described, but the remaining eleven (nearly 16 per cent) have a different complex. These last have only three chromosomes in the small group, the two members of the small pair and one normal-sized member of the second pair (Figs. 2 and 3), but have an additional chromosome in the second group, which comes in the size series among the members of the third and fourth pairs. Study of the first and second spermatocytes shows the additional chromosome in this group to be the synaptic mate of one of the small chromosomes in the first group. The size relationship of the members of this unequal pair is shown most strikingly in side views of second spermatocyte anaphases (Fig. 10).

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[2] Unfortunately, Dr. R. L. King, in a paper dealing with three species of *Trimerotropis*, has used the name of a subgenus, *Pseudotrimerotropis*, as the name of the genus. Such a procedure is not justified, as will appear from the following statement. McNeill (1901), in his "Revision of the Genus *Trimerotropis*," arranged the species of *Trimerotropis* in two subgenera, *Agonozoa* and *Trimerotropis*. Rehn (1901), pointed out that McNeill had placed the type species of *Trimerotropis* in his subgenus *Agonozoa* and used *Trimerotropis s.s.* for another subgenus. He added, "It is quite apparent *Agonozoa* is a synonym for restricted *Trimerotropis* and a new name is necessary for McNeill's subgenus *Trimerotropis*. To supply the deficiency I propose *Pseudotrimerotropis*." Kirby (1910) in his "Catalogue" used all three subgenera as genera. Caudell (1911), in a critical review of Kirby's catalogue, states: "*Pseudotrimerotropis* Rehn, of which *Trimerotropis vinculata* may be taken as the type, is based on characters which are not, in the reviewer's opinion, of generic importance and the genus should be sunk in synonymy under *Trimerotropis*." The following statement by Rehn is appended as a footnote to Caudell's article: "The name *Pseudotrimerotropis* was proposed to replace the restricted *Trimerotropis* of McNeill, true *Trimerotropis* being equal to his *Agonozoa*. The author of the name has never considered it of more than subgeneric rank."

Lateral views of first spermatocytes show that the tetrad formed by this pair may divide either equationally (Fig. 5) or reductionally (Fig. 6). A count of over 300 division figures in individual numbered 1571 gave 90 per cent of the former to 10 per cent of the latter. An equation division in the first spermatocyte is, of course, followed by a reduction division in the second (Fig. 9), while a reduction division in the first entails an equation division in the derived second spermatocytes. The last are of two sorts in regard to the pair under consideration. Those which receive the small homologue are identical to the seconds in typical individuals; for example, compare the two small dyads in Fig. 12, which is from specimen No. 1571 where the homologues are unequal, with those in Fig. 11, from an individual where these homologues are equal in size. The other sort have but one small dyad; the second is replaced by the large homologue as is shown in Plate II, Fig. 15. (This figure is also from individual No. 1571.)

Another occasional condition sheds light on the variation in time of segregation of this pair and also on one of the functions of synapsis; namely, that it is a mechanism which insures segregation which, otherwise, does not necessarily occur. When unequal, these homologues at times come into the first spermatocyte metaphase as separate chromosomes; whether they synapse and separate before the metaphase or do not synapse at all is not known. A count of 52 metaphases in another specimen (No. 1927) gave eight in this condition. Such first spermatocytes contain thirteen chromosomes, three of which, the accessory and the unsynapsed, unequal homologues, are dyads. Any one of four results may occur as a consequence of such a condition. 1. One dyad may go to each pole undivided. This is, in effect, a segregation division and gives second spermatocytes of the usual types. 2. Both may go to the same pole undivided. Thus, in effect, an entire tetrad goes into one cell while the other lacks any representative of these homologues. All chromosomes in both cells are dyads and will divide in the ensuing division. Inevitably, certain sperm will carry chromosome number 2 in duplicate and should any of these fertilize an egg, the resultant zygote would necessarily be triplicate in respect to the factors carried by this chromosome. 3. Both may divide as shown in Fig. 7. This is in effect an equational division, but each of the resulting second spermatocytes receives two monads (Figs. 13 and 14) which are incapable of division at this time; one monad may pass into each spermatid, both of which would consequently contain a full complement of chromosomes, or both monads may pass into the same spermatid. The result in the last case is the same as in "2," above. 4. One may divide while the other passes undivided to one pole. Figure 8 shows the larger dyad in an equational division, while its small homologue is going undivided to one pole. One of the four spermatozoa derived from such a first spermatocyte would again contain this chromosome in duplicate; one would lack it completely; and two would be normal. All of the described conditions have been found except the converse of that shown in Fig. 8, where the small dyad divides and the larger one segregated, and I have little doubt that such complexes exist, also. One should bear in mind that while a tetrad can undergo two divisions without any

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EXPLANATION OF PLATES

The complexes were drawn with the aid of a camera lucida at a magnification of 2800 diameters. They were reduced $\frac{1}{3}$ in reproduction.

The chromosomes under especial consideration on each plate are in solid black. All lateral views of entire complexes, except Fig. 12, are from two and sometimes three sections.

Explanation of Plate I

(*Trimerotropis citrina*, entire complexes)

Fig. 1. Spermatogonium, polar view, too small pairs, 19 large chromosomes, (9 pairs and the accessory).

Fig. 2. Spermatogonium, polar view, one small pair. The synaptic mate of the third small chromosome is one of the 20 large chromosomes of which there are 9 pairs, the accessory, X, which is unpaired, and one medium-sized unpaired chromosome. The chromosome set off by the dashed line is in the adjoining section but is obviously the homologue of the similar one near it.

Fig. 3. Similar to the last (all chromosomes in one section).

Fig. 4. First spermatocyte, side view, homologues equal; 60 of the 71 individuals studied were of this type. Figures 1 and 11 illustrate the spermatogonia and second spermatocytes, respectively, which are characteristic for these specimens.

Fig. 5. First spermatocyte, side view, homologues of one small pair unequal; from same specimen as spermatogonium shown in Fig. 3. Eleven out of 71 individuals are of this type. Division of unequal pair equational.

Fig. 6. Similar to Fig. 5. Division of unequal pair reductional.

Fig. 7. First spermatocyte anaphase. Unequal homologues not synapsed. Both dyads dividing, in effect an equational division.

Fig. 8. First spermatocyte anaphase from same specimen as Fig. 6. Dyads of unequal pair not synapsed; The larger dyad dividing equationally, the smaller segregating.

Fig. 9. Second spermatocyte, polar view, derived from a first spermatocyte division such as is represented by Fig. 5.

Fig. 10. Same type as preceding, side view.

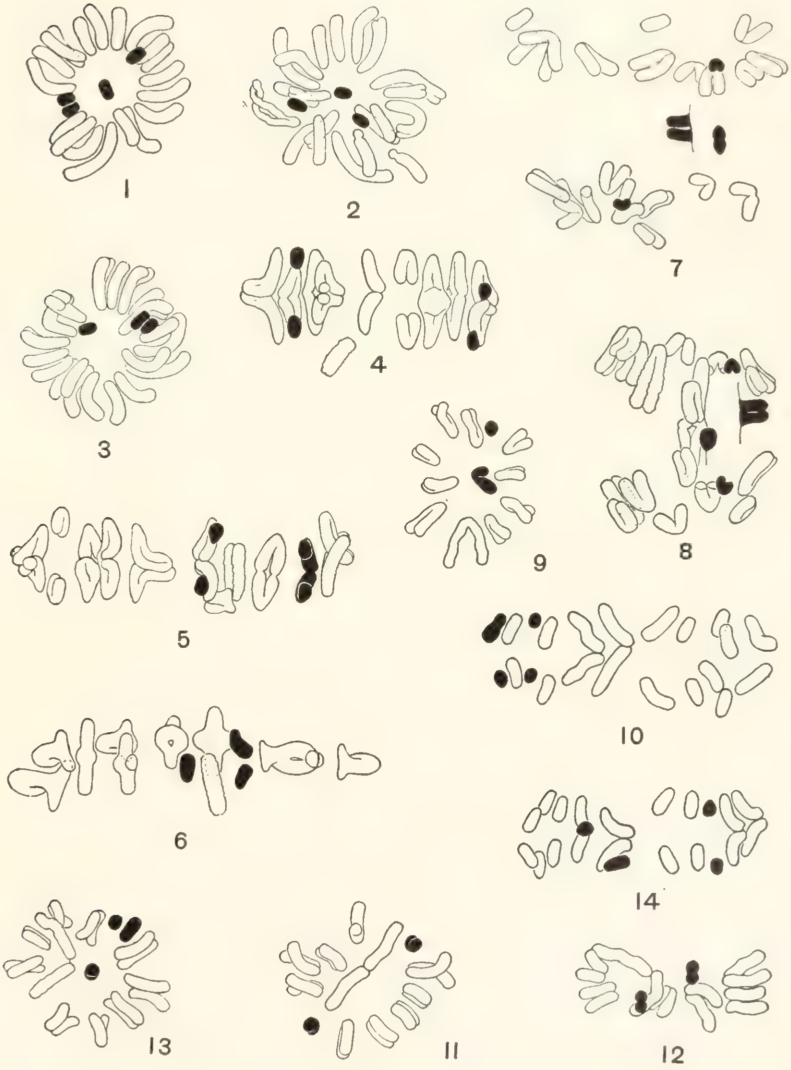
Fig. 11. Second spermatocyte, polar view, from one of the 60 typical specimens.

Fig. 12. Second spermatocyte, side view, composition of small dyads identical to those in preceding cell but derived through segregation of unequal homologues in the first spermatocyte. Drawings from same individual as Figs. 2 and 15. This cell received the smaller homologue, that shown as Fig. 15 the larger one.

Fig. 13. Second spermatocyte, polar view, (type with accessory). Thirteen chromosomes, two of which are monads, derived from such a first spermatocyte as that shown in Fig. 7.

Fig. 14. Second spermatocyte (type lacking accessory), side view, entire complex. Unequal homologues not synapsed. Both monads derived from such a first spermatocyte as is shown in Fig. 7.

PLATE I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extensive reconstruction, a dyad can divide only once, and a monad cannot divide at all but must simply pass to one pole.

In three of the 71 specimens of this species studied the smallest pair also is composed of unequal homologues. But, the members of this pair ordinarily segregate in the first maturation division (roughly 95 per cent of the observed instances) in contrast to about 90 per cent post-reduction shown by the pair just described. In this respect the smallest pair approaches the unequal pair in *Brachystola magna* (Carothers, 1913).

Figures 16 to 19, inclusive, are from an individual in which the members of both small pairs are unequal. Figures 16 and 17 are incomplete lateral views of first spermatocyte complexes. The first shows both unequal tetrads dividing equationally, the latter the smaller pair dividing reductionally. Figures 18 and 19 are from second spermatocytes and show the segregation which follows an equational division of the first spermatocyte, such as is represented in Fig. 16. In case segregation occurs in the first division, as illustrated in Fig. 17, the second spermatocytes show a simple equational division of dyads with like chromatids.

EXPLANATION OF PLATE II

FIG. 15. Second spermatocyte, polar view, only one small dyad. The homologues of the unequal tetrad segregated in the preceding division and this cell received the larger component.

FIG. 16. Part of first spermatocyte complex, side view, showing both small tetrads unequal and dividing equationally.

FIG. 17. From same individual. Smallest tetrad dividing reductionally.

FIG. 18. Part of second spermatocyte metaphase, side view, members of smallest pair dividing reductionally.

FIG. 19. Similar to preceding, both small pairs dividing reductionally.

FIG. 20. Lateral view, first spermatocyte anaphase, *Mecostethus gracilis*. Homologues of both small pairs unequal. The members of one pair have segregated; those of the other pair have divided equationally.

FIG. 21. Slightly earlier stage than the preceding, both of the unequal tetrads dividing equationally.

FIG. 22. Second spermatocyte, polar view, same species. Segregation division for unequal homologues of both small pairs.

FIG. 23. First spermatocyte anaphase, side view, *Amphitornis bicolor*, both small tetrads unequal and dividing equationally.

FIG. 24. Second spermatocyte, side view, from same species showing reductional division of the two small pairs.

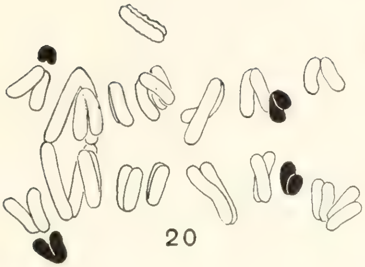
FIG. 25. Sister second spermatocyte metaphases, *Amphitornis bicolor*, side view, one dyad caught in plate at preceding division. One monad free in each cell.

FIG. 26. Unequal tetrads in late prophase, *T. citrina*. Newly acquired portion more dense.

FIG. 27. Same in *Mecostethus gracilis*. Note the tendency for the newly acquired portion to organize itself into chromomeres.

FIG. 28. Late prophase tetrads *T. citrina*, showing chromomere vesicles.

PLATE II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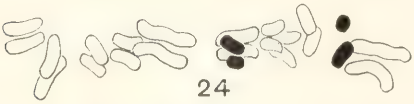
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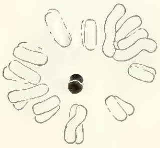
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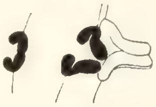
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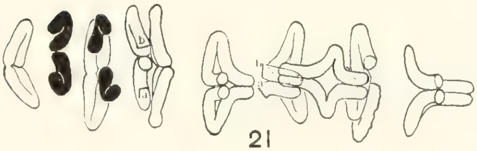
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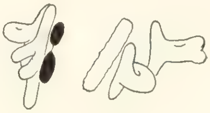
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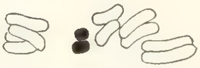
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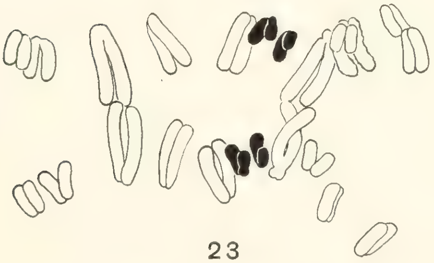
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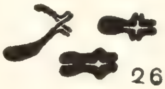
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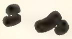
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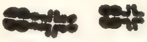
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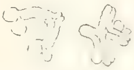
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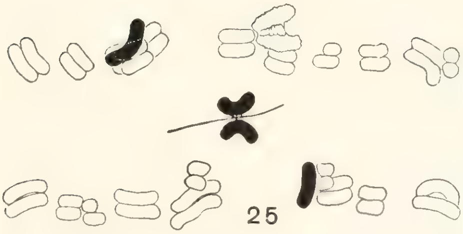
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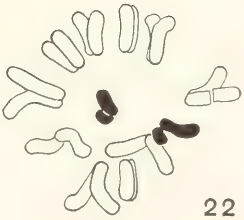
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Let us consider briefly the other two species. (2) *Mecostethus gracilis:* This species along with others of this genus has been studied in detail by Prof. C. E. McClung and I shall refer to it only in regard to the point in question.

The first five males to be added to our collection were taken at Salisbury Cove, Maine, in the summer of 1923. All show the second pair unequal and two out of the five have both small pairs unequal. The specimens taken near Cheboygan, Michigan, are of especial interest, for while some of them are of the types just mentioned, others supply a link which although not unknown in other species, has been lacking hitherto for both *M. gracilis* and *T. citrina*; namely, they are of the theoretically expected type with the second tetrad composed of two large homologues. While this condition automatically throws the tetrad out of its usual place in the size series, it is still readily recognized by certain peculiarities.

As to time of segregation, the homologues of both pairs when unequal may undergo either pre- or post-reduction. A count of 150 cells from three consistent individuals gave a ratio of pre-reduction to post-reduction for the second pair of 1:8, while the ratio for the first pair derived from a count in 63 cells was 1:12, which is not very different from that for the second pair. The numbers were not extended because counts from another specimen demonstrated that the range of variation from individual to individual is such that ratios may have no significance. Both small tetrads in this specimen are unequal and no attempt was made to distinguish between them further than to ascertain that both varied as to time of segregation. A count in 55 cells gave a combined ratio of approximately 1:1. Evidently either one or both of these tetrads in this individual behaves differently from either of them in the other three individuals. A closer study might reveal a corresponding morphological variation such as Wenrich (1916) found for tetrad C in *Phrynotettix.* Drawings from *M. gracilis* are shown on Plate II. Figure 20 is from a side view of a first spermatocyte anaphase. One pair was dividing equationally, the other reductionally. Figure 21 is a similar view from a slightly earlier stage in which both pairs were dividing equationally. Figure 22 is a polar view of a second spermatocyte metaphase illustrating post-reduction for both pairs.

(3) *Amphitornis bicolor:* Individuals of this species also may have either one or both of the two smallest pairs unequal. Our material is interesting for several reasons: in the first place certain individuals were collected by Dr. W. S. Sutton at Russell, Kansas, over 30 years ago, others were taken by myself in 1919 and 1921 at three widely

separated localities in Kansas and in the vicinity of Pike's Peak in Colorado, so that the range of this material both in area and time is considerable.

The unequal homologues in this species show a much greater predisposition than in either of the others towards post-reduction. Prereduction occurs in not over 1 per cent of the cases noted.

Figure 23 is a lateral view of a first spermatocyte anaphase and Fig. 24 a similar view of a second spermatocyte metaphase; both show post-reduction for these two pairs.

2. *Chromomere Vesicles and the Origin of These Unequal Homologues.*—Most cytologists who have studied unequal homologues have reported, without very satisfactory evidence, however, that the inequality was due to a loss of chromatin by one member of the pair. This doubtless is the cause sometimes, but I believe that in the three species concerned in the present paper all of the inequalities described are due to increases; and, furthermore, these increases are not fictitious ones due to chromatin which belongs to some other chromosome having been acquired by these enlarged homologues. I am convinced that the increase is brought about by the transformation of terminal granules into chromomere vesicles which become densely chromatic and are maintained. An idea of the process may be gained from Plate II, Figs. 26, 27 and 28. Vesicles such as are shown in the last figure take a dense chromatic stain in earlier stages when the chromatin threads stain very lightly. Gradually, as the threads take a more dense stain the vesicles become less densely stained until at one point the vesicles are seen to be filled with a flocculent chromatin which is the same in appearance, practically, as that in the chromatin thread. (These observations have been verified by the use of Feulgen's nucleal reaction which gives results similar to iron-hæmatoxylin for chromomere vesicles.) Ordinarily, the vesicles continue to lose their staining capacity until they become indistinguishable. That they really may be persistent structures, however, is indicated by the fact that they have been recognized in both metaphases and early anaphases. Certainly, in spermatogenesis they are always associated in the prophases with definite chromomeres of particular chromosomes (Carothers, 1913, p. 498). Wenrich was the first to note that these vesicles may be terminal instead of subterminal. He wrote (1916, p. 113), "Are polar granules to be classed in the same category as the plasmosomes (chromomere vesicles)? Is it possible for a polar granule to become transformed into a plasmosome and then back into a polar granule again? The last question seems to be answered in the affirmative by the conditions in *Phrynotettix.* In the case of B, for example, one of the proximal

granules becomes "expanded" in only about 16% of the cases counted. In becoming expanded it has become a plasmosome. When it is not expanded, it remains a polar granule." Wenrich regarded the larger homologue as the normal type and believed that a loss of chromatin had occurred and, hence, did not suspect that this "expanded" condition might lead to a permanent enlargement. I believe this, however, to be the logical conclusion, since growth of chromosomes involves a similar mechanism. After each cell division, the chromosomes become diffuse or "expanded"; in many instances, they form chromosomal vesicles; when they again condense, they have grown back to a size characteristic for each; that is, they have doubled their mass during the period of diffusion and are now ready for another division.[3]

Whatever the function of chromomere vesicles, granting, as seems likely, that they have some specific function, the associated effect is, in some instances, a prolonged period of diffusion (*e.g.*, *Phrynotettix*) which may well lead to a differential increase of chromatin for the chromomeres concerned.[4] The same mechanism may function equally well to secure reduction in size of chromomeres if the period of condensation is prolonged and the period for growth (expansion) consequently shortened or, as is usually the case, the periods of condensation and diffusion may be so balanced as to maintain a constant size. Figure 27 from *M. gracilis* is of particular interest as it indicates that these enlarged terminal vesicles may be organized finally into chromomeres. See also McClung, 1928. Plate XXVIII, Figs. 51*d*, 53 and 56. As already mentioned, in certain members of this species from Michigan both homologues of the second pair are of the large type. The tetrad formed by such a pair loses its usual position in the size series but is still recognizable as it forms a ring instead of the V which is characteristic for tetrads of this genus.

If my suggestion is correct, then new chromatin has been organized to form a permanent component of the complex; and, granted that the chromatin bears hereditary factors, a mechanism for progressive changes is shown. In other words, if the chromomere vesicles characteristic of certain chromosomes are capable of a permanent modification in kind or amount of chromatin in response to a changed environment with its resultant need for an altered metabolism, these changes would be adaptive in character.

[3] W. E. de Mol (1927), in reference to *Hyacinthus orientalis*, states that the chromosomes probably receive material from nucleoli either at permanent secondary constrictions or by satellites which are really nucleolar globules not taken up in the chromosomes.

[4] Gertrand Hasse-Bessell (1928) believes that the satellites are differentiated portions of chromosomes, organs for assimilation of chromatin, and that the satellite is the morphological expression of chromatin absorption on its active surface.

Attention should be called to the fact that in the Orthoptera the only homologous chromosomes which have been found to be unequal in size are those which form the two or three smallest tetrads and these are precisely the ones which are usually characterized by chromomere vesicles.

3. *An Accident During Mitosis, A. bicolor.*—Ten or twelve second spermatocytes in one individual show a dyad caught in the "zwischenkörper," resulting from the first spermatocyte division, with one chromatid in each daughter second spermatocyte (Plate II, Fig. 25). The abnormality is due, apparently, to some slightly pathological state which existed at the time of the first spermatocyte divisions and seems worthy of record as a morphological condition which may explain occasional aberrant genetical ratios. My expectation was that the trapped dyad in each case would be found to belong in one or the other of the second spermatocytes concerned, and that one of them would possess a normal dyad derived from the same tetrad. Drawings of entire complexes show, however, that each of the affected seconds actually have their normal number of chromatids, since there is a monad in each equatorial plate which, together with the monad of the trapped dyad in the corresponding cell, accounts for the components of the expected dyad. Figure 25 shows the chromosomes of two second spermatocytes derived from one such first. The upper complex contains eleven dyads including the accessory, which is easily recognized by its roughened outline and one monad. The lower complex contains ten dyads and one monad. A small segment of the boundary between the two cells is represented together with the trapped dyad. In the drawings the two equatorial plates are placed nearer together and consequently nearer the median cell boundary than they actually are for the sake of economy of space on the plate.

Stages later than that shown were not available, but the monads in the equatorial plates would behave, doubtless, as other monads in similar situations; that is, each would pass to one pole without division so that of the four spermatids derived from these two cells, two would be normal and the nuclei of the other two would lack one chromosome with the result that an egg fertilized by one of the latter would be haploid for this chromosome. The additional possibility exists that the monad left at the cell boundary may form a chromosomal vesicle and migrate to join with the pronuclei in fertilization, in which case the production of individuals triploid for this chromosome would follow fertilization by any sperm which received both monads.

4. *Incipient Octad Formation—T. citrina, individual No. 4853.*—This specimen, in addition to being one of the eleven *T. citrina* which

have an unequal pair of homologues, exhibits a tendency on the part of two non-homologous pairs to form a multiple. On account of the latter peculiarity, this specimen yields interesting evidence on three points: (1) an unusual mode of octad formation; (2) the behavior of spindle fiber attachments; and (3) the time of segregation. The individual is one of fifteen taken at Kingman, Kansas, and is the only one of the 71 *T. citrina* studied which shows any tendency toward multiple formation. The insect seems to be typical externally and the testes are normal.

The occurrence of octad multiples was predicted by McClung (1905). They were first recognized by Woolsey (1915) in *Jamaicana*, and Robertson (1916) correctly suggested that the three atelomitic rings in the first spermatocytes of *Stenobothrus* and *Chloëaltis* were octads. They have since been found in *Hesperotettix*, *Circotettix*, *Stauroderus*, and a subtropical genus, *Sphenarium*. The number of multiples is constant for species and perhaps even genera in *Stenobothrus*, *Chloëaltis* and *Circotettix*. McClung (1917) has shown that, on the other hand, in *Hesperotettix viridis* there is considerable variation within the species, but constancy for any given individual. The specimen of *T. citrina* under consideration is unique in that it shows variation from cell to cell within the individual, ranging in the first spermatocytes from no multiple through an octad with one homologue of each tetrad united by their distal ends to those where both pairs are so associated. This attachment at the distal ends is another point of difference between this multiple and those previously described where the union occurs at the ends associated with the spindle fibers.

The two pairs concerned are of intermediate size, one somewhat larger than the other, as may be seen in the spermatogonium figured (Plate III, Fig. 29).[5] Here two non-homologous chromosomes (solid black) are united at their distal ends. One is perhaps one-fourth longer than the other. The homologue of each is free and indistinguishable among the other members of the complex. This is the only clear spermatogonial complex which was obtained, though some cells seem to show the full complex of 23 chromosomes and others to have two multiples resulting in 21 chromosomes.

The first spermatocytes show much variation in the behavior of these two tetrads. The more usual condition is for them to form a ring-shaped octad (Plate III, Figs. 30 and 35*d*). Its structure is easily understood by comparison with the two tetrads represented in solid color in Fig. 31. This is a drawing of one of the few first spermatocytes where no octad has been formed. If the free ends (the ends not associated with the spindle fibers) of these tetrads are joined and the fiber attachments remain as they are in the separate tetrads, the result is the octad ring.

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[5] These unequal homologues segregate in either division as in the preceding instances. They are not inked in solid in this case as the peculiar behavior of the two pairs concerned in octad formation is emphasized in this manner.

Morphologically, this ring differs widely from the octads of *Stenobothrus*, *Hesperotettix* and *Chloëaltis*, in which the open part of the ring lies in the plane of the spindle and where there are but two places for fiber attachments. In the present instance the open part of the ring lies in the plane of the equator and there are four loci for fiber attachments (two for each of the component tetrads). In *Circotettix*, as I pointed out in 1921, the octad often is indistinguishable from the atelomitic tetrad. As to the origin of these three different types of rings; the atelomitic tetrads have come about, apparently, from a shift of fiber attachment. The octads of *Circotettix*, *Hesperotettix*, and probably those of *Stenobothrus* and *Chloëaltis* have been formed by union at the ends to which the fibers attach, while in this individual the union has occurred at the distal ends, and fiber attachments have remained constant though in certain cases those on one of the tetrads do not function.

This brings up the other forms the octad may assume. Next to the ring the most common form is that where the tetrads are united by one arm only as seen in Figs. 32, 35*a*, 35*c*, 35*e* and 36. Such forms are the logical descendants of spermatogonia similar to the one represented in Fig. 29 where one homologue of each pair is free. For this type one can say with assurance that segregation occurs in the second division. See Figs. 39 and 43, second spermatocyte anaphases, where an inverted V comparable to the V in the spermatogonium, Fig. 29, has segregated from the two free homologues which were not identified in the spermatogonia. This is the only form which this multiple assumes where one can be certain as to which is the segregation division. One who did not know the synaptic relations might conclude that whatever type of division was occurring in the multiples represented in Figs. 33 and 34, the opposite type was represented in Figs. 32, 35*a*–*e*, and 36, but this assumption is not justified because the chromatids of the individual tetrads have been through a period of parasynapsis; consequently, it is not possible to say which chromatids are derived from different parents.

The third form in point of frequency of occurrence is like the first in that the homologues of the two tetrads are united at the ends which ordinarily would be free, but the dyads of the smaller tetrad are not united with each other (Figs. 33, 34 and 35*b*). As to the origin of such forms, the synaptic ends of the small pair may be unable sometimes to get together as a result of their position at the ends of the longer pair so that what synapsis occurs is merely a continuation of that of the latter and must proceed in a direction reverse to normal. This, I am inclined to believe, is the explanation of such forms; although they may be due to a separation of the smaller homologues after synapsis. The end result is the same in either case. The fibers on either the larger or smaller (but not on both) of the tetrads function in such octads. Figure 33 is of interest as evidence of a struggle for supremacy between the fibers to the two tetrads. While those of the smaller tetrad have gained the ascendancy, there is a distinct torsion of the arms of the larger tetrad; indeed, from this figure there might be doubt as to whether the long arms would not yet swing into the equator and the division occur in the opposite plane. The similar octad shown in Fig. 34, however, has reached a stage where there is no reasonable doubt that the division will occur in the plane indicated in Fig. 33.

A fourth type (Fig. 35*f*), although found only once, is of especial interest, since such a division would give an entire tetrad to each

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Explanation of Plate III

Trimerotropis citrina, specimen No. 4853
(Multiple solid black)

Fig. 29. Spermatogonial complex, polar view. Two non-homologous chromosomes united at their distal ends.

Fig. 30. First spermatocyte, polar view, octad multiple in form of ring.

Fig. 31. First spermatocyte, polar view, 12 chromosomes. Components of octad ring shown in 30 appear as two separate tetrads.

Fig. 32. First spermatocyte, side view, members of octad united by only one arm of each. Entire complex not shown.

Fig. 33. First spermatocyte, side view, not complete. Members of octad united by both arms, dyads of smaller tetrad separated but retaining their fiber attachments.

Fig. 34. Similar to preceding.

Fig. 35. Various forms which the octad assumes in the first spermatocytes. Fig. 35*f* would result in segregation of entire tetrads.

Fig. 36. Similar to Fig. 32.

Fig. 37. Second spermatocyte, polar view, entire complex. Multiple such as would be derived from octads shown in Figs. 33 and 34.

Fig. 38. Second spermatocyte, polar view, entire complex, derived from such a first spermatocyte as is shown in Fig. 31.

Fig. 39. Second spermatocyte, side view of anaphase, entire complex. Multiple derived from forms like those shown at Figs. 32, 35*a*, 35*e* and 36.

Fig. 40. Polar view of such a second spermatocyte multiple in metaphase.

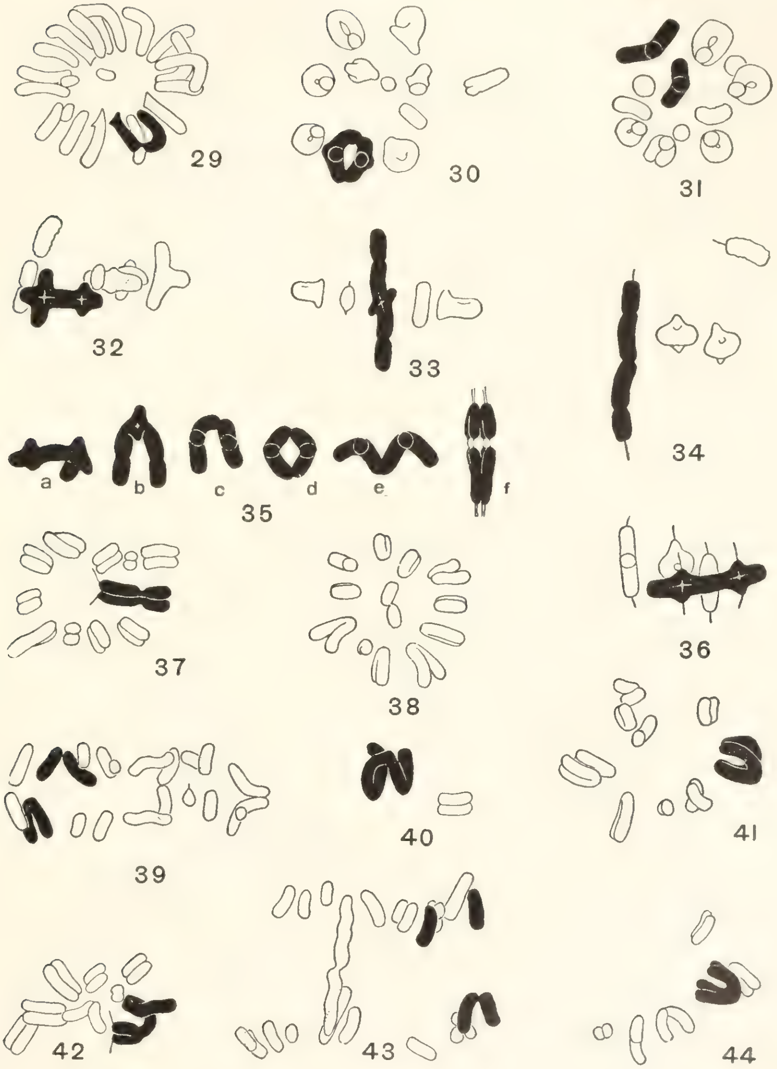
Fig. 41. Second spermatocyte, polar view, multiple derived from ring octads like those shown in Figs. 30 and 35*d*.

Fig. 42. Second spermatocyte metaphase, oblique view, multiple, a modification of type shown in preceding figure.

Fig. 43. Entire complex similar to that shown in Fig. 39 except that this one contains the accessory while the other lacks it.

Fig. 44. Partial complex similar to one shown in Fig. 41.

PLATE III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derived second spermatocyte while all four of the resulting spermatozoa would lack one member of the normal haploid series and be duplex for another. If such spermatozoa should fertilize eggs, the new individuals would be haploid for one member of the series and triploid for another, while the number of chromosomes would remain normal. The structure of the octad may be understood by assuming that an octad similar to the one illustrated in Fig. 30 rotated 90° about an axis passing through the points of union of the two tetrads in a plane vertical to the equatorial plate. All of the normal spindle fiber attachments were functioning.

In the second spermatocytes, one finds an unusual series of forms which would be very puzzling without a knowledge of the variations occurring in the first spermatocytes. Figure 38 illustrates the ordinary 12-chromosome form which results as one of the daughter cells from the division of such a first spermatocyte as that represented in Fig. 31. The form of multiple which is shown in Fig. 37 may be derived from a first spermatocyte multiple like that in Fig. 35*b* with only the fibers to the larger member acting in this division as in the first, or from those of the type figured in 33 and 34 with the fibers to the larger component, which were in abeyance at the first division, functioning. In any case, half of the normal spindle fibers have failed to operate, in counter-distinction to the remaining instances where, in both divisions, all of the fibers normal for the two separate tetrads have functioned, although half of them would have been sufficient for the necessary distribution of the parts of the octad.

Figures 39 and 43 show entire complexes in anaphase; one lacks, the other possesses, the accessory. The multiple in both is of the type which results from an association of only two dyads, one from each tetrad, giving an inverted V to one pole and two free rods to the other. The appearance of such a multiple in metaphase is shown in Fig. 40. Figures 41 and 44 show the multiple derived from first spermatocytes like those illustrated in Figs. 30 and 35*d* where both ends are united. The result, in this division, is an inverted V to each pole.

To summarize:

1. This multiple is not constant for the individual. When present, it is formed by union of the distal ends of the chromosomes, in this respect resembling certain *Oenothera* multiples rather than other Orthopteran octads.

2. Ordinarily each chromatid of a chromosome has a functional spindle fiber. In this octad, instances occur where only half of them function, *e.g.*, Figs. 33, 34 and 37. Figure 33 indicates competition between the two sets.

3. Second spermatocytes show clearly both pre-reduction, Fig. 37, and post-reduction, Figs. 39 and 43.

IV. Discussion

Let us consider the four sources of information as to time of segregation mentioned at the end of section I. (1) Sex chromosomes, as already shown, may undergo either pre- or post-reduction, but one or the other is constant for any particular species.

(2) *Heteromorphic Homologous Chromosomes.*—So far as the unequal tetrads are concerned, similar conditions exist in other species in our collection, but the observations here presented together with those previously published, Carothers, 1913; Wenrich, 1916; Robertson, 1916, are believed to be sufficient to demonstrate the range from 100 per cent pre-reduction to 100 per cent post-reduction. In heteromorphic pairs (Carothers, 1916, 1921), where the difference is associated with spindle fiber insertion, segregation has been found to occur only at the first maturation division. Probably the reason for this is to be found in the mechanical conditions involved. Certainly, we should not assume from this that when these tetrads are composed of homomorphic dyads they also segregate at the first division.[6]

(3) *Polyploidy.*—In *Œnothera* and *Datura* mutants where the normal complex has become unbalanced, the extra dyads are reported to segregate at the first maturation division. But to demonstrate that such behavior is invariable would require a very detailed examination. On the other hand, Lesley and Frost, 1928, reported, "additional chromosome fragments," in two extreme "small" *Matthiola* plants. In both plants these "fragments" (supernumaries?) segregate at either division. This behavior agrees with that of the unsynapsed dyads reported in this paper which are shown also to segregate at either division.

(4) *Genetical Evidence.*—Allen, 1924, from a study of inheritance of non-sex-linked characters in the four clones of *Sphærocarpos* derived from one pollen mother cell concluded that, "in some way qualitative segregation can be brought about in both divisions."

Whiting, 1924, concluded that in the parasitic wasp, *Habrabracon* (*Hadrobracon*), "The first maturation division of the egg may be either equational or reductional for various loci apparently according to chance." His data was obtained from females heterozygous at four loci.

Similarly, Goldschmidt and Katsuki, 1928, in a combined cytological and genetical study of a mosaic gynandromorph strain of *Bombyx*

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[6] For additional discussion of segregation of sex-chromosomes and heteromorphic homologues, *see* Carothers, 1926.

mori, showed that a non-sex-linked recessive gene for skin transparency may segregate at either maturation division.

Briefly, then, both cytological and genetical data justify the following conclusion: Reduction in number of chromosomes should not be confused with the segregation (reduction) division which applies only to individual pairs. Reduction in number of chromosomes is brought about by synapsis, while segregation of the parts of the tetrads derived from one parent from those derived from the other results from the two maturation divisions which follow each other in rapid succession and together separate the four chromatids of each tetrad into different cells.

LITERATURE LIST

References to the earlier papers may be found in the excellent bibliographies in both E. B. Wilson's, The Cell in Development and Heredity, MacMillan, 1925, and in E. L. Mark's paper listed below.

Allen, C. E., 1924. Inheritance by Tetrad Sibs in Sphærocarpos. *Proc. Am. Phil. Soc.*, **63**: 222.

Belling, J., and A. F. Blakeslee, 1922. The Assortment of Chromosomes in Triploid Daturas. *Am. Nat.*, **56**: 339.

Carothers, E. Eleanor, 1913. The Mendelian Ratio in Relation to Certain Orthopteran Chromosomes. *Jour. Morph.*, **24**: 487.

Carothers, E. Eleanor, 1921. Genetical Behavior of Heteromorphic Homologous Chromosomes of Circotettix (Orthoptera). *Jour. Morph.*, **35**: 457.

Carothers, E. Eleanor, 1926. The Maturation Divisions in Relation to the Segregation of Homologous Chromosomes. *Quart. Rev. Biol.*, **1** (3): 419.

Caudell, A. N., 1911. Some Remarks on Kirby's Synonymic Catalogue of Orthoptera, Vol. 3. *Ent. News*, **22**: 158.

Goldschmidt, R., and K. Katsuki, 1928. Cytologie des erblichen Gynandromorphismus von Bombyx mori L. *Biol. Central.*, **48**: 685.

King, R. L., 1923. Heteromorphic Homologous Chromosomes in Three Species of Pseudotrimerotropis (Orthoptera: Acrididae). *Jour. Morph.*, **38**: 19.

Kirby, W. F., 1910. A Synonymic Catalogue of Orthoptera. 3: British Mus. Nat. Hist.

Lesley, Margaret M., and Howard B. Frost, 1928. Two extreme "Small" Matthiola Plants etc. *Am. Nat.*, **62**: 22.

Mark, E. L., 1881. Maturation, Fecundation and Segmentation of Limax campestris, Binney. *Bull. Mus. Compar. Zool., Harvard Coll.*, **6**: 173.

McClung, C. E., 1905. The Chromosome Complex of Orthopteran Spermatocytes. *Biol. Bull.*, **9**: 304.

McClung, C. E., 1917. The Multiple Chromosomes of Hesperotettix and Mermiria (Orthoptera). *Jour. Morph.*, **29**: 519.

McClung, C. E., 1928. Differential Chromosomes of Mecostethus gracilis. *Zeitschr. f. Zell. u. Mikr. Anat.*, **7**: 756.

McNeill, J., 1901. Revision of the Orthopteran Genus Trimerotropis. *Proc. U. S. Nat. Mus.*, **23**: 393.

Rehn, J. A. G., 1901. Random Notes on North American Orthoptera. *Trans. Am. Ent. Soc.*, **27**: 334.

Robertson, W. R. B., 1916. Chromosome Studies, I. *Jour. Morph.*, **27**: 179.

St. George, v. la Valette, 1865. Über die Genese der Samenkörper, Erste Mittheil. *Schultz Arch.*, **1**: 403.

St. George, v. la Vallette, 1876. Über die Genese der Samenkörper, Vierte Mittheil. *Schultz. Arch.*, **12**: 797.

Van Beneden, E., 1875. La maturation de l'oeuf, la fécondation et les premières phases du développment embryonnaire des mannifères etc. *Bull. Acad. Roy. de Belgique*, **44**: 686.

Van Beneden, E., 1883. Recherches sur la maturation de l'oeuf et la fécondation. *Arch. de Biol.*, **4**: 265.

Van Beneden and Ch. Julin, 1884. La Spermatogénèse chez l'Ascaride Mégalocéphale. *Bull. Acad. roy. des Sci. des Lettres et des Beaux-Arts de Belgique*, **7**: 312.

Waldeyer, W., 1888. Ueber Karyokinese und ihre Beziehungen zu den Befruchtungsvorgängen. *Arch. mikr. Anat.*, **32**: 1.

Wenrich, D. H., 1916. The Spermatogenesis of Phrynotettix magnus, etc. *Bull. Mus. Compar. Zoöl., Harvard Coll.*, **60**: 55.

Whiting, P. W., 1924. Some Anomalies in Habrobracon and their Bearing on Maturation, Fertilization and Cleavage. *Anat. Rec.*, **29**: 146.

Woolsey, Carrie I., 1915. Linkage of Chromosomes Correlated with Reduction in Numbers etc. *Biol. Bull.*, **28**: 163.

THE SIZE OF THE BODY AND THE SIZE OF THE ENVIRONMENT IN THE GROWTH OF TADPOLES

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INTRODUCTION

Body size is influenced to varying degrees by environmental conditions in the several phyla of organisms. Among aquatic animals, in general, it has long been known that adult size and rate of growth are functions of the volume of the water available as well as of the more usually limiting environmental factors of food supply and temperature. In the mammals it is generally assumed that the average rate of growth is the optimal rate of growth, and that mean adult body size represents the product of internal regulatory functions. But in poikilothermic animals it is apparent that body size is equally controlled by conditions. The readiness with which the body size of these animals responds to external factors presents an opportunity for experimental analysis of some of the size regulators.

Numerous qualitative observations upon the growth of tadpoles in crowded and uncrowded situations have indicated that there exist marked effects of the size of the environment upon body size at any given age (Pflüger, 1883; Yung, 1885). The natural assumption was that the effects were due to deleterious substances accumulating in the medium. But the experiments of the above investigators, and of Bilski (1921) and Goetsch (1924), threw grave doubts upon this view, and indicated that a truly spatial influence was at work. A chemical influence seemed merely pathological, but a physical influence seemed worthy of experimental analysis. It was for the purpose of discovering the nature of this spatial factor and its quantitative effectiveness that the following measurements were undertaken.

METHODS

For the objective in mind, the best criterion of body size was the body weight. This was measured by weighing one or more individuals in a manner which gave strictly comparable results. All weighings were done in duplicate.

Tadpoles above 100 milligrams in weight were separated from the water by pouring the latter into a sieve. The food material and the debris were then allowed to remain in the sieve while each tadpole was picked up with a perforated spoon and transferred to a dish of clean water. All the tadpoles were then sieved together from the clean water and emptied onto filter paper, from which they were poured into a tared weighing bottle.

Smaller tadpoles were usually too delicate to endure the draining on filter paper. They were poured into a tared Gooch crucible, the crucible was drained and wiped thoroughly and then put into the weighing bottle. For tadpoles less than 20 milligrams each in weight, it was necessary to have in addition a tared amount of water within the weighing bottle. For duplicate weighings the draining and taring were repeated. When the tadpoles were drained in a Gooch crucible, more water clung to the tadpoles than when the tadpoles were drained on filter paper. A small correction for this water could be made by inference from older tadpoles that could be weighed both with and without the crucible. Cultures of less than four individuals could be weighed with sufficient accuracy only after sizes of 40 milligrams or more had been attained.

For embryos not yet hatched from the gelatinous membranes, body volumes were estimated from microscopic micrometer measurements of two diameters. The specific gravity of the embryos was assumed to be 1.04; Bialaszewicz (1908) found that the specific gravity gradually changed from 1.08 to 1.03 during the development of frog eggs up to the time of hatching.

The plan of the experiments was to keep the tadpoles under as uniform conditions as possible. All eggs in comparable experiments were derived from a single clutch or brood, and all clutches of *Rana pipiens* were collected in one small stream. It is believed that paternity as well as maternity is uniform within each clutch. Tap water was the medium used throughout the experiments, the water usually being allowed to stand in large bottles before use, so that when used all comparable cultures received uniform samples of water at the proper temperature. Food, consisting of *Spirogyra, Vaucheria,* and other algæ as collected, was supplied in such amounts that it was always available to the tadpoles. A certain number of living and dead small animal bodies were available as food in this material, and when any of the experimental tadpoles died they were usually eaten by the survivors. For the most part the tadpole cultures were in pyrex dishes; but in every case dishes of the same size and material were furnished to comparable cultures. Most of the cultures were maintained under constant temperature conditions. These conditions were secured by keeping each dish, covered, in a small room which was cooled by air drawn from an adjacent refrigerator room by a fan that went into action whenever the atmosphere attained a certain temperature. In this way for months at a time the temperature of the water was kept at 19.0° ± 0.2° C.

The chief observations were made on *Rana pipiens*; but supplementary measurements were carried out on *Rana sylvatica,* which is characterized by markedly different absolute body sizes.

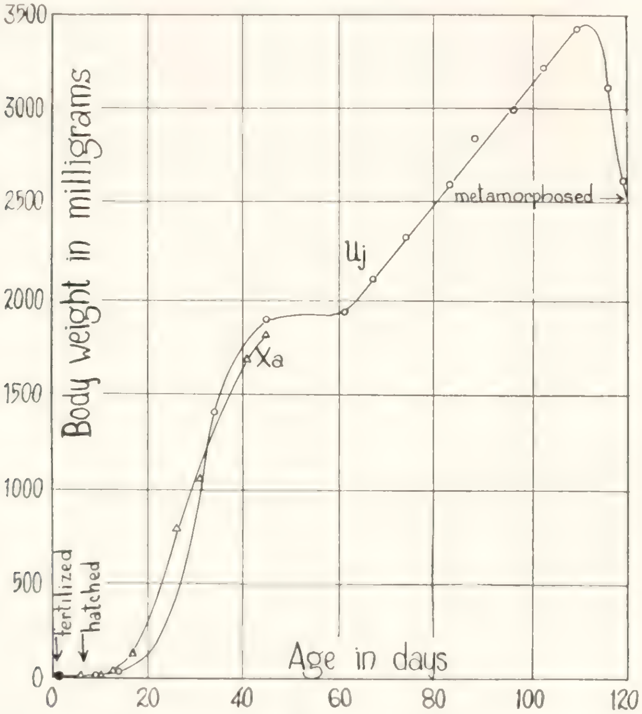

Fig. 1. Growth in weight of *Rana pipiens* at 19° C. under optimum conditions. Within the first fifteen days after fertilization the body weight was determined by weighing 64 individuals together; thereafter single isolated individuals were weighed. Individual *Uj* began development on April 9, 1929; individual *Xa* began on April 25, 1930, and was voluntarily discontinued on June 9, 1930.

Normal Growth Curve

No curve of growth at constant temperature is known for any species of amphibian. For this reason the increases of body weight with time under the optimum conditions of the present experiments are presented. It must be understood that under other conditions, such as with another food, or in still larger aquaria, or with another race of *Rana pipiens,* the rate of growth may be quite different.

The body weight throughout the entire life span is represented in Fig. 1 for a single individual, from fertilization until after metamorphosis. This individual (*Uj*) grew in a volume of 500 cc. of water that was changed weekly. Before hatching from the egg membranes, tadpoles increase in weight only very slightly; this brief period has been accurately studied by Bialaszewicz (1908). Thereafter body weight increases rapidly for two or three weeks; it is believed by Krizenecky (1917), Fauré-Fremiet (1923) and others that during a large part of this period growth may proceed without food. Thereafter percentage growth increments decrease markedly. This decrease is probably due to adverse conditions of unknown kind, since all the cultures were retarded at the same time. But actually no individuals of *Rana pipiens* have been cultured without relatively slow growth for some weeks before the maximum weight was attained preceding metamorphosis. This point will be mentioned again. Metamorphosis is marked by a stoppage of weight increase and then a sudden loss of over half the body weight. The loss of weight is one of the first sharp signs of metamorphosis that can be detected. The changes at metamorphosis will be discussed in the next paper.

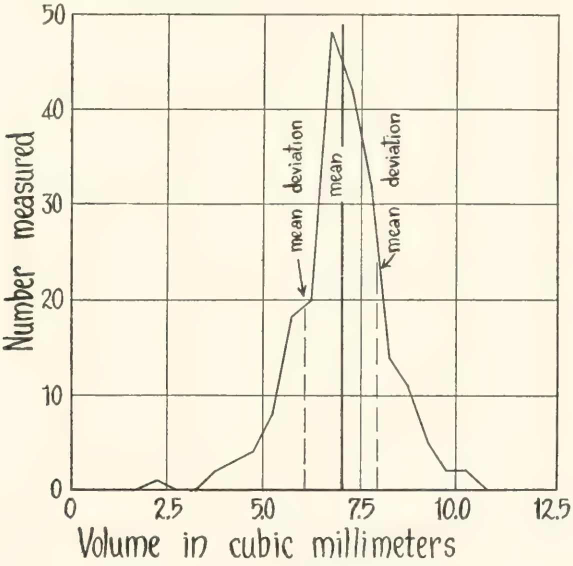

Fig. 2. Frequency distribution of embryo volumes measured on brood *E*. Each of the 212 individuals, in the yolk-plug stage, was measured with an ocular micrometer in two diameters; the volume of each was calculated as length × square of breadth × $\pi/6$.

The present experiments are concerned chiefly with growth up to 1000 milligrams. Figure 1 shows that the rates of growth were not identical under the optimum conditions in broods *U* and *X*.

The first point on the growth curve, which is the egg weight, was always determined not by measurement of the single egg but by measurement of a large sample of eggs from the clutch. A frequency distribution for egg volume is obtained which is always approximately normal, as is shown for one large sample in Fig. 2. Only small differences in mean embryo volume (less than 25 per cent) were found between the first cleavage stages and late gastrula stages. This conclusion may also be drawn from the data of Bialaszewicz (1908) on *Rana temporaria,* though some data of Morgan (1906) seem to indicate a significant increase of total volume during blastocoele formation.

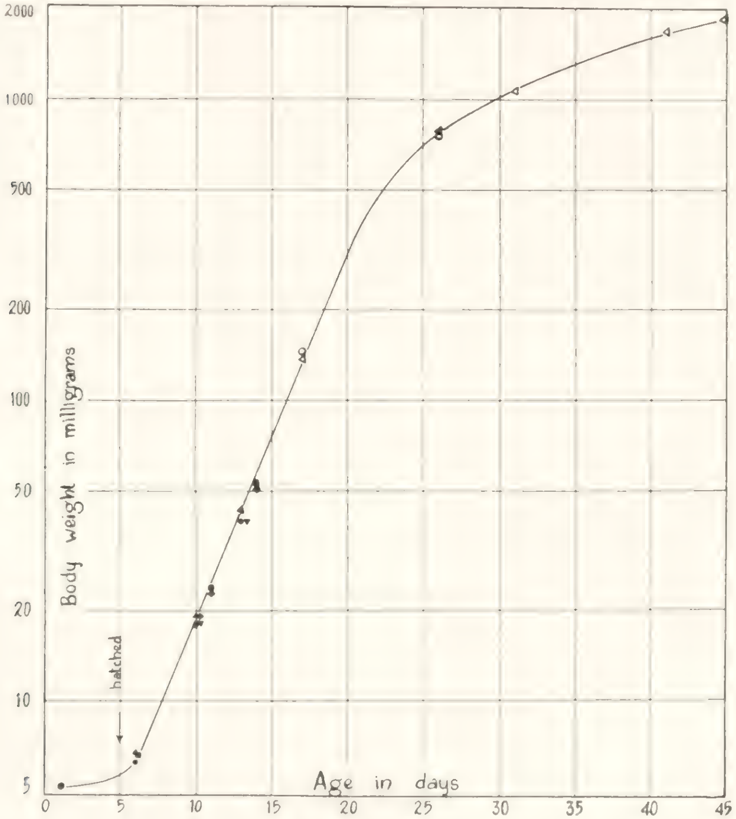

FIG. 3. Growth in weight of brood X at 19° C. under optimum conditions. Weight is plotted, as in all the subsequent charts, on a logarithmic scale. Solid points represent 64 or 32 individuals weighed together; open points, single individuals isolated in 1000 cc. of water.

Altogether over 800 embryos of *Rana pipiens* have been measured, with a mean weight of 5.87 milligrams. Significant differences occur between broods of *Rana pipiens* from the same pond, the extreme broods of the ten broods measured having means of 3.30 and 8.35 milligrams.

It was shown by Halban (1910) and Terroine (1921) that egg sizes are determined in considerable part by the sizes (or ages) of the parents. Chambers (1908) believed that the size of the individual egg was of great consequence in the future growth of the individual, but his data were hardly conculsive on this point.

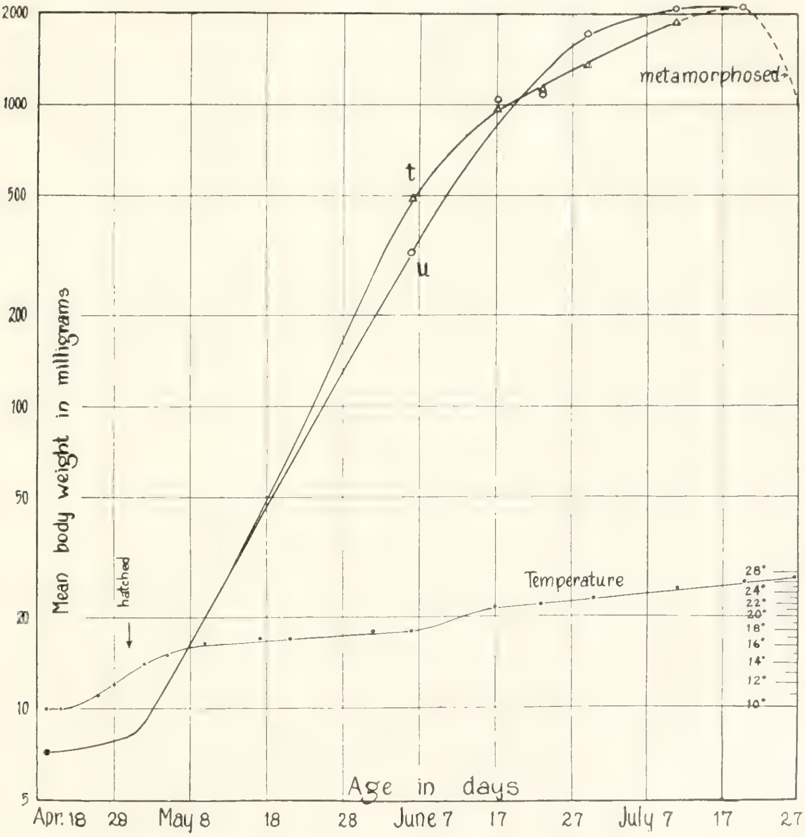

Fig. 4. Growth in weight of *Rana pipiens* in the wild. Most egg-laying in the season of 1927 occurred on April 18th in this pond. Group *t* represents tadpoles sampled from a circumscribed region of the small stream in question; Group *u* represents tadpoles sampled from the whole stream 500 yards in length. Each weight plotted is the average of 4 to 11 newly collected individuals for *t* and of 15 to 46 individuals for *u*. The noon-day temperatures of the water are plotted also.

A curve for optimal growth (*Xa*) is plotted upon a logarithmic scale of body weights in Fig. 3. The most interesting feature of this method of representation is that in most cultures a certain region of the graph of growth is a straight line. " Logarithmic growth " begins at hatching of the embryo and continues under the best conditions at 19° C. for two weeks. During this period the body weight doubles every two and a half days. Thereafter the percentage increment in weight falls off continuously, though it is sometimes possible to find another straight line on the logarithmic scale lasting from three until about six weeks after hatching. The logarithmic scale emphasizes the early parts of the growth process; it minimizes to the eye the contrasts in weight that will be presented below.

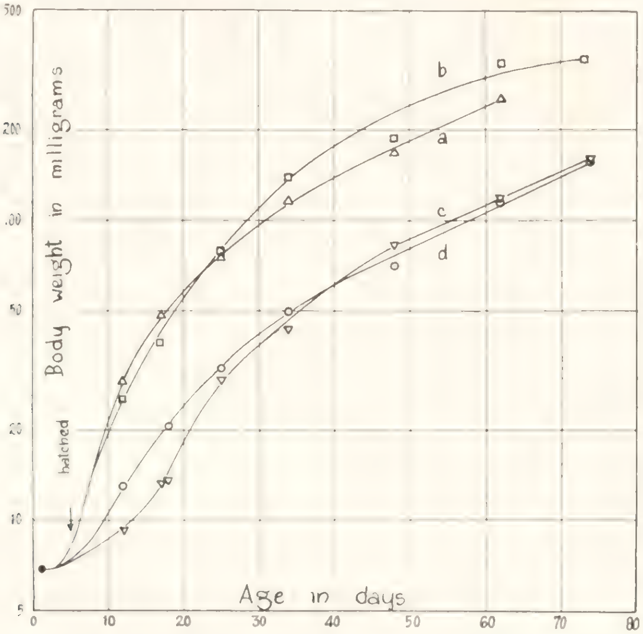

FIG. 5. Growth in weight of brood *C* at two different temperatures. Each group or culture contained 32 individuals; *a* and *b* were kept at 17° ± 1° C., *c* and *d* were kept at 9.5° ± 1° C.

It is generally considered that ideal growth is logarithmic; that increment of body substance should every day be proportional to the substance already present. The maintenance of the logarithmic rate for the two week period at 19° C. is remarkable because of the fact that during this period the percentage water content of the tadpoles undergoes huge changes, as is known from the data of Davenport (1897), Galloway (1900), Schaper (1902), Bialaszewicz (1912) and others. The decreased logarithmic rate thereafter might be easily pictured in terms of Herbert Spencer's (1866) conception that some limiting factor becomes inadequate to keep up with the mass requirement, perhaps a factor of intake or of elimination of some substance. It may be said that such a factor prevents much further enlargement of the tadpole body, but that after metamorphosis new factors are at work so that logarithmic growth begins again, judging from the meagre data on the growth of the adult frog summarized by Donaldson (1911).

Although logarithmic increases of weight are found in many kinds of organisms, and although logarithmic scales have been used in graphing the present data, it must be stated that there is no intention of emphasizing those portions of the growth curves in which the logarithm of the body weight is linear. Some of the data, as those in Fig. 5, may be accurately represented as parabolic functions. The truth is that the data are not sufficiently reproducible under diverse conditions of food and activity, and in diverse broods and species of tadpoles, to insure that any one formula, or any one controlling factor that it implies, is innately characteristic of the organism studied. The expenditure of ingenuity in fitting formulæ to the present data is not justified, because of the fact that growth of an organism is the average of many cycles of mitotic and incretory activity in the several organs and tissues of the body.

Although no curves for growth in Amphibia were previously worked out at constant temperature, it is worthwhile to compare Fig. 3 with those for growth in *weight* that have been reported. *Rana temporaria* (=*fusca*) as studied by Schaper (1902) showed, in spite of progressively rising temperatures, a progressive falling off in percentage weight increment from the time of hatching. *Bufo americanus,* studied by Miller (1909), showed rather an increase in the relative increments for an entire month after hatching. But here it is likely that the temperatures, though not recorded, rose more rapidly. It is apparent also that the food supply and the aquarium space were more favorable in Miller's cultures than in Schaper's. Similar results were obtained on *Amblystoma* in natural ponds by Dempster (1930). The data on *Amblystoma* of Patch (1927) and on *Diemyctylus* of Springer (1909) are insufficient for comparison.

Some data upon the growth of *Rana pipiens* in nature were obtained in the course of the present experiments. Samples of all the tadpoles in the particular stream from which all the eggs for the laboratory observations have been collected were taken at weekly intervals during one season. It should be stated that no eggs, tadpoles, or frogs taken from this pond were ever identified as being other than *Rana pipiens.* The curve shown in Fig. 4 is primarily influenced by the temperature of the stream. So far as could be observed, the food supply was plentiful throughout the season, though the population of the pond was large.

The effect of temperature upon the rate of growth was studied only in a preliminary manner in the laboratory. Two cultures (Fig. 5) were kept in a small undercooled air-bath at 9.5° C. within a refrigerator room, and were comparable in every respect with two cultures kept in another air-bath at 17° C., this bath being cooled by a jacket of flowing water. Throughout the course of growth the former were about half the weight of the latter, from which it may be concluded that assimilation per unit of mass was half as rapid only in the first week after hatching. To maintain half the weight later on, however, meant doubling the body weight in the same time interval at both temperatures.

THE EFFECT OF CROWDING ON GROWTH

It was noticed by numerous observers, beginning with Hogg (1854) and Semper (1873), that individuals of various aquatic species were retarded in growth by confinement in small dishes or by the presence of many other individuals. It was ordinarily supposed that this influence was due to fouling of the water by products of metabolism. This explanation did not seem to fit all the facts, though it has recently been again urged by Crabb (1929); and for Amphibia the suggestion was made by Pflüger (1883) that mechanical disturbance was a hindrance to growth. Yung (1885) experimented with deep and shallow aquaria of uniform volume, and concluded that surface area of the water was the primary factor. It may be pointed out that tadpoles kept in deep vessels are stimulated to greater activity by the shortage of oxygen, and that the path of locomotion, which is usually horizontal, is shorter. Babák (1906) noticed that crowded tadpoles were not only smaller but had relatively smaller digestive tracts, a conclusion which is not fully confirmed by the more extensive measurements of Elven (1928). Bilski (1921) and Goetsch (1924) tested various devices for overcoming the possible effect of chemical disturbances.

It seemed that the first step in the analysis of this spatial factor lay in the accurate measurement of growth rates under conditions of crowding. For this purpose, body weights were determined at frequent intervals in a number of cultures derived from a common brood. The cultures were varied in two ways: (1) in number of individuals per unit volume of water, and (2) in volume of water per given number of individuals.

The first mode of comparison is illustrated in Fig. 6. Each culture here represented was kept at 19° C. in a pyrex dish with 500 cc. of water which was changed weekly. The water had a surface of 280 sq. cm. and therefore a depth of 1.8 cm. The retardation in growth is such that the average weight in the culture containing 64 individuals was, at 19 days after hatching, only one-sixth, and at 28 days after hatching only one-twelfth the average weight in cultures of one single individual. Differences of this sort require no elaborate methods for their demonstration. Four other series of the same sort, grown during three separate seasons, confirm both qualitatively and quantitatively the result of Fig. 6. Each series represents two months of care and measurement.

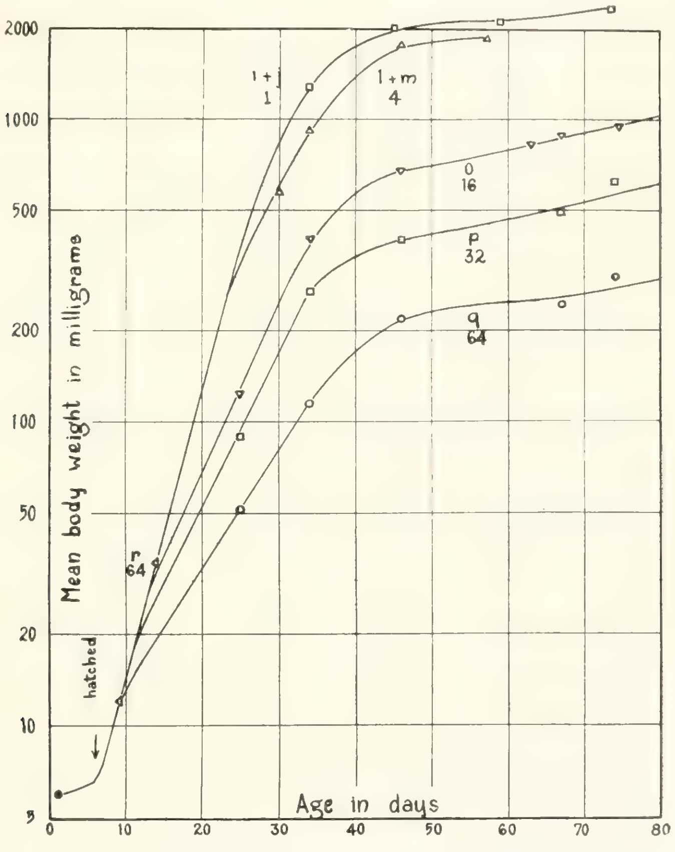

Fig. 6. Influence of density of population upon growth in weight of brood *U* at 19° C. Each culture was in 500 cc. of water, changed weekly, and fed *ad libitum*. Numbers indicate the individuals per culture. In the less crowded conditions two duplicate cultures were averaged.

The effect of volumes of water of varying sizes is illustrated in Fig. 7. In these cultures the proportion of surface to volume of water was nearly constant, so that only the one factor varied. The smallest aquarium was so small that practically all the space was occupied by the food supply, and the tadpoles were ultimately killed by fouling of the medium.

Even the increase of the volume of medium from 500 cc. to 1000 cc. for one tadpole had a marked effect upon body size. The effect of large volumes (or few tadpoles) became more marked at advanced stages; the effect in the range of small volumes manifested itself earlier.

The *shape* of the growth curve was altered by crowding (Fig. 6). Measurements made early in the life cycle (in brood *X*) showed that for the first week after hatching the rates of growth were equal in all cultures. Then, however, the most crowded ones lagged behind and thereafter proceeded at much slower rates than the isolated ones. Less crowded ones began some days later to lag, and proceeded thereafter at intermediate rates. The isolated or single individuals were the last to depart from the logarithmic rates of growth, and in most instances continued at rates greater than any others of the brood.

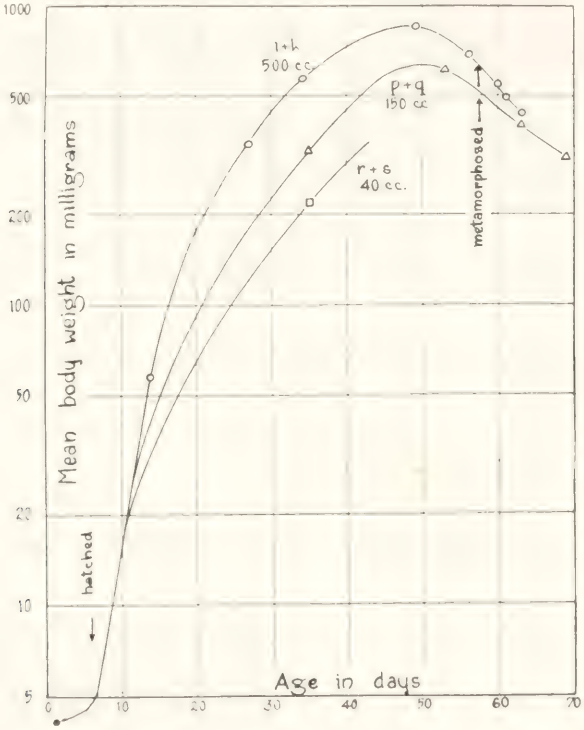

FIG. 7. Influence of size of culture dish upon growth in weight of *Rana sylvatica*, brood *Q*, at 19° C. Each culture contained four individuals and occupied the volume of water indicated, being changed weekly and fed *ad libitum*. Two duplicate cultures were averaged throughout.

These comparisons may also be made quantitatively. The most direct method is to plot the mean body weight (*W*) upon a given day against the number of individuals per culture (n). It is then found by trial, as shown in Fig. 8, that the best correlation is between the logarithm of the body weight and the square root of the number present. In other symbols,

$$\log W = a - b\sqrt{n} = a - \frac{b}{\sqrt{v}}.$$

The volume of medium per individual (v) is the reciprocal of the number of individuals per volume (n). The values of the constants *a* and *b* depend on the particular age at which the comparison is made, since they are functions both of absolute size and of the relative rates of growth. Both constants increase with age. A somewhat different formula was employed by Bilski (1921). If the growth curve could be represented by a single equation, it would be feasible to have values of a and b to hold for all ages. But it is obvious that the growth curve itself depends in fact upon the increasingly severe effect of crowding as the tadpoles grow older. The inhibition of growth by crowding is manifested only in proportion as the tadpoles occupy more and more considerable portions of their medium.

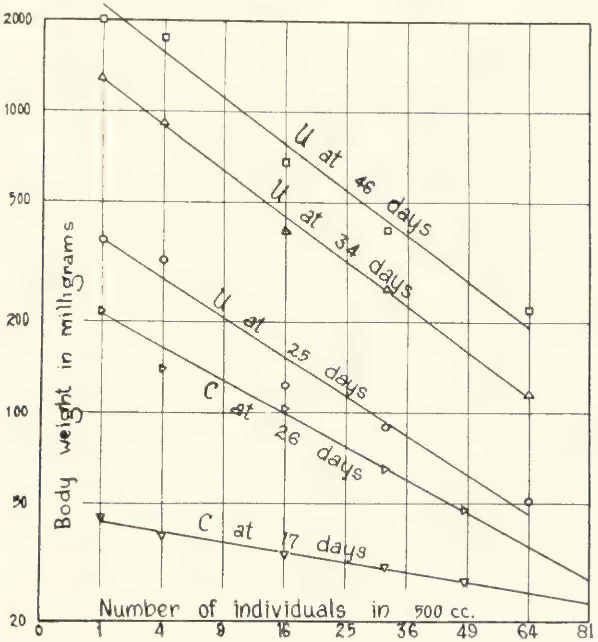

FIG. 8. Correlation of body weight attained with number of individuals per culture. The number of individuals is plotted upon a square root scale. The brood U data are taken from Fig. 6 at 19° C.; the brood C data were obtained at inconstant room temperatures, which were, however, alike for all cultures.

Mechanism of the Crowding Effect

Knowing to what extent diminution of the size of the environment affects tadpoles at various ages, diverse conditions were tested to find how the crowding has its influence upon growth. To rule out each factor in turn required subjection of crowded and uncrowded animals to experimental devices which are now to be mentioned in more or less logical order. The conclusions have been previously summarized in abstract (Adolph, 1929).

1. *Isolation After Crowding.*—Individuals that had been in crowded cultures were isolated singly or in pairs at various ages in culture dishes of the same size. These individuals immediately assumed rapid rates of growth, gaining in percentage weight much faster than uncrowded individuals of the same *age,* and almost equalling the earlier performances of uncrowded individuals of the same *size.* Examples may be seen in Fig. 11, p. 366. Such isolated tadpoles showed the same subsequent decreases in growth rate as the uncrowded controls, once the size at which logarithmic growth usually ceases had been reached.

2. *Growth in Old Medium.*—The water in which many tadpoles had been crowded was taken, either every day or every week, and given to similarly crowded individuals of the same age and brood. The old water gave slight inhibition of growth compared to the fresh-water controls in most cases. But the significant result was that the inhibition was very slight.

In other tests the water from crowded individuals was given to single individuals of the same brood; one such experiment is shown in Fig. 9. These again were only very slightly retarded in growth compared to controls in fresh water, and the influence was significant only in water from very crowded cultures where metabolic substances may have accumulated considerably.

Individuals that had grown in fresh medium but in crowded groups were isolated in dishes where they were alone but were in old medium. They began to grow rapidly just as did their controls that were isolated in fresh medium. These tests proved that nothing added to or subtracted from the water by the presence of other tadpoles was responsible for the chief inhibitory influence of crowding. It seemed to mean, further, that no chemical condition whatsoever was responsible for the influence. Nevertheless, further tests were made to rule out other sorts of chemical conditions.

3. *Crowding with Other Broods.*—Groups of two or four individuals of a younger brood or with numerous individuals of another amphibian species. The younger brood of the same species (*R. pipiens*)

had slightly less inhibitory effect than had equal numbers of the same brood, but there was no doubt of their effect. Other species used to furnish crowding were *Rana sylvatica* and *Amblystoma punctatum.* The *sylvatica* tadpoles were as effective as equal-sized *pipiens* tadpoles. The *Amblystoma* larvæ gave inconclusive results because they were not furnished with sufficient animal food and so did not grow appreciably. As a result, the experimental *sylvatica* tadpoles grew almost as rapidly as their uncrowded controls.

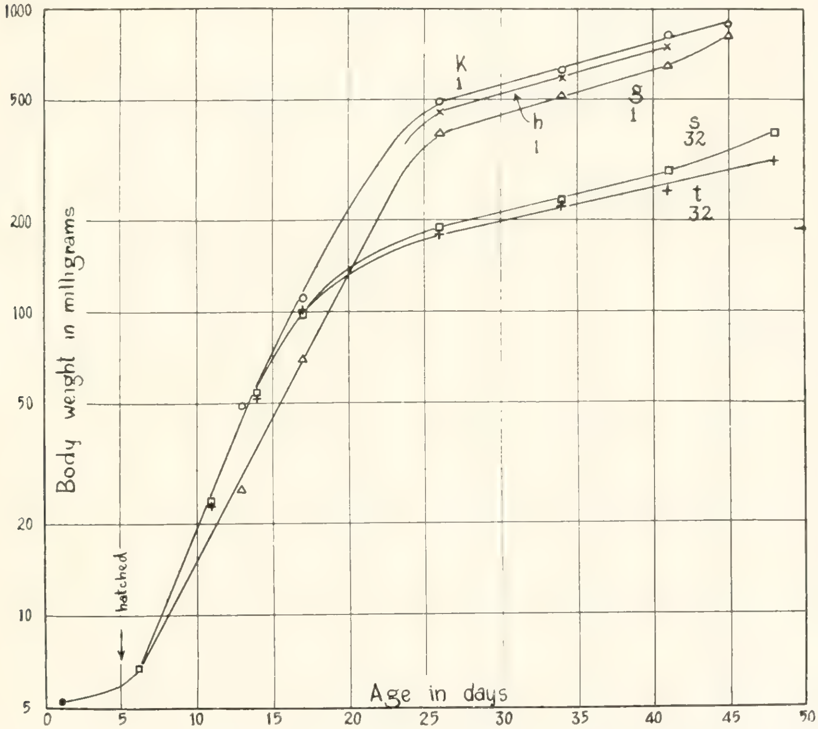

FIG. 9. Comparison of growth in fresh water and in water previously inhabited by tadpoles. Cultures *s* and *t* each contained 32 individuals; every four days their water (500 cc.) was given to *g* and *h* respectively, each containing one individual. Culture *k* contained one individual which received 500 cc. of fresh water every four days. All belonged to brood *X* at 19° C.

In one series of tests a number of snails or of leeches were placed with the tadpoles. These tadpoles grew just as rapidly as their controls of the same brood.

In another experiment an uncrowded individual that had grown large was placed in the same culture with 32 crowded individuals of the identical brood. Growth was checked in the large individual. The small crowded ones were already growing so slowly that the additional crowding was not significant.

4. *Confinement.*—It has already been noted that reducing the size of the culture-dish had the same effect as increasing the number of individuals in the dish. Thus, even a single individual was greatly retarded if his environment was small. Experiments were now done in which the volume of water remained large but the animal was confined to a small portion of it. This was done by suspending a cheesecloth bag in one liter of water so that the animal could move through only about 300 cc. of the water. Growth was markedly retarded as compared with the growth of animals having a whole liter of water in which to move, as Fig. 10 shows. Animals were later exchanged between bag and no-bag containers. As can be seen in the weight chart, the previously retarded one now grew faster than its control in another bag; while the newly retarded one lost weight for a time, subsequently gaining only as fast as its confined control.

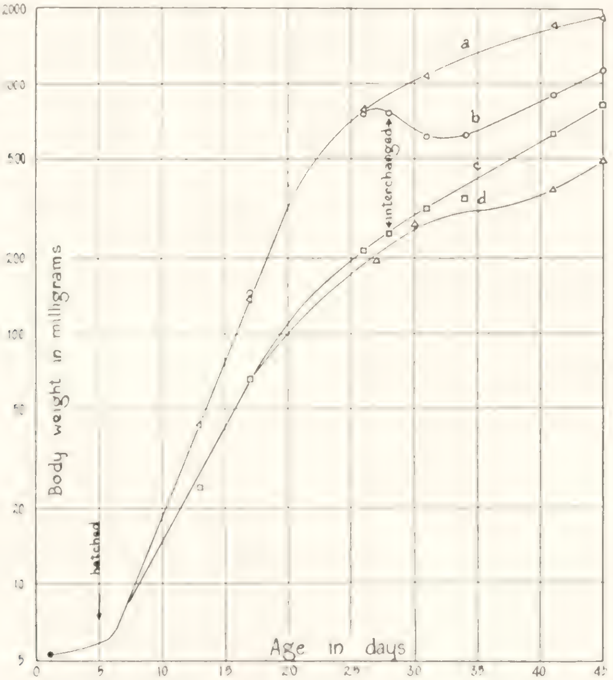

Fig. 10. Influence of reducing the free space in a large volume of water upon growth at 19° C. Each of the four cultures contained one individual of brood *X* in 1000 cc. of water; but in *c* and *d* a cheesecloth bag confined the individual to about 300 cc. of that water. On the 28th day individuals *b* and *c* were interchanged so that *b* was now confined. Thereupon *b* was retarded in growth while *c* forged ahead.

Again the conclusion is indicated that not the volume of medium but the volume through which the animal can move unimpeded is the effective factor.

5. *Aëration.*—An obvious possibility, upon the hypothesis that crowding was a chemical influence, was that lowered oxygen tension or increased carbon dioxide tension prevailed in the crowded cultures.

No effect of surface area of the water could be found within the comparatively narrow limits tested. Single individuals in one liter of water grew at the same rate when that water had a surface area of 133 sq. cm., as when it had an area of 530 sq. cm. and was therefore only one-fourth as deep.

In another series of cultures the water was continuously aërated by a stream of bubbles from the compressed-air supply. In them the contrast was as great as usual between crowded and uncrowded individuals. Such tests seem to rule out any volatile substances as essential to the crowding effect.

6. *Addition of Various Substances.*—In a few tests the tadpoles were cultured in media having various materials added to the tap water. Water in which frogs had previously been for a day or more invariably killed the tadpoles. This fact contrasts with the harmlessness of tadpole excreta; the acidity of frog urine may have been the important factor. Small concentrations of urea and of sodium iodide were tested, but the experiments were not carried on long enough to demonstrate any effects upon the growth with and without crowding. No differences could be observed in similar cultures whether in pyrex glass or in soft glass, nor when extra glass was immersed in the water.

7. *Concentration of Food.*—It seemed possible that the higher concentrations of food that were necessary for maintenance in crowded cultures were deleterious. In certain uncrowded cultures equally large excesses of food (green algæ) were supplied, but no inhibition of growth was observed. The presence or absence of rarer kinds of food, such as minute animals, with the green food apparently had no influence. To certain crowded and uncrowded cultures liver from various sources was added as a supplement to the vegetable diet without result. The action of any disintegration products of the food, which were not large in amount, are ruled out because the food was replaced more rapidly than it died.

8. *Frequency of Renewing Medium.*—A number of the same possible factors are eliminated from consideration by tests in which the tap water and food were changed more or less frequently. Cultures containing few and many individuals were changed daily, in contrast with cultures containing the same numbers changed weekly, as was the rule

in nearly all the tests. In all cases, as shown in Fig. 11, a slight superiority of size was attained by those whose medium was renewed daily. This result agrees with that of tests in which previously occupied water was given to the tadpoles, in showing that substances given off by tadpoles have demonstrable, but only very slight, inhibitory influences upon growth.

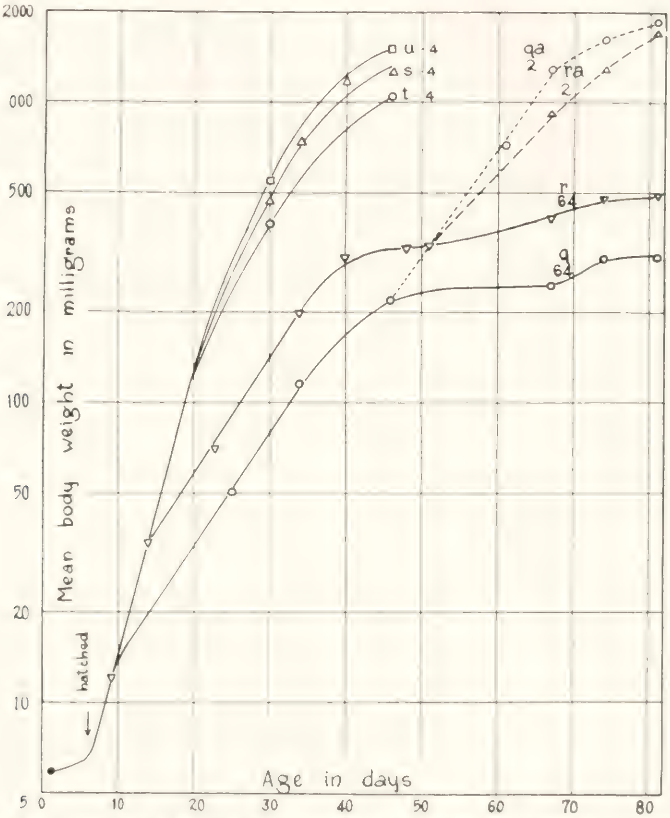

FIG. 11. Influence of the frequency of changing the medium upon growth in weight, in brood *U* at 19° C. in 500 cubic centimeters. Cultures *s*, *t*, and *u* contained four individuals each; cultures *q* and *r* contained 64 individuals each. Of these *s* and *r* had fresh water daily; and the others only weekly. Upon the 46th and 51st days respectively, two individuals from each of cultures *q* and *r* were isolated in 500 cc. and so were able to resume rapid growth. The water in *ra* was renewed daily, in *qa* weekly.

Medium occupied much longer than one week, especially by intermediate-sized tadpoles, usually became obviously foul, and when actually allowed to remain it killed the tadpoles. It might be assumed, though it is not proven, that any lethal materials would retard growth in concentrations too weak to kill. It is surprising therefore to find that they retard exceedingly little as they do.

9. *Flowing Water.*—The crucial test for many factors of growth was to culture the tadpoles in running water. Individuals in varying numbers were placed in cheesecloth sacks which would just fill 1.5-liter beakers, the open tops of the sacks being supported by wires above the beakers so that water could not overflow the edges. The sacks were held spread out by frames made of glass rods. Water was renewed in each beaker at rates of 300 to 600 cc. per minute, day and night, for several months.

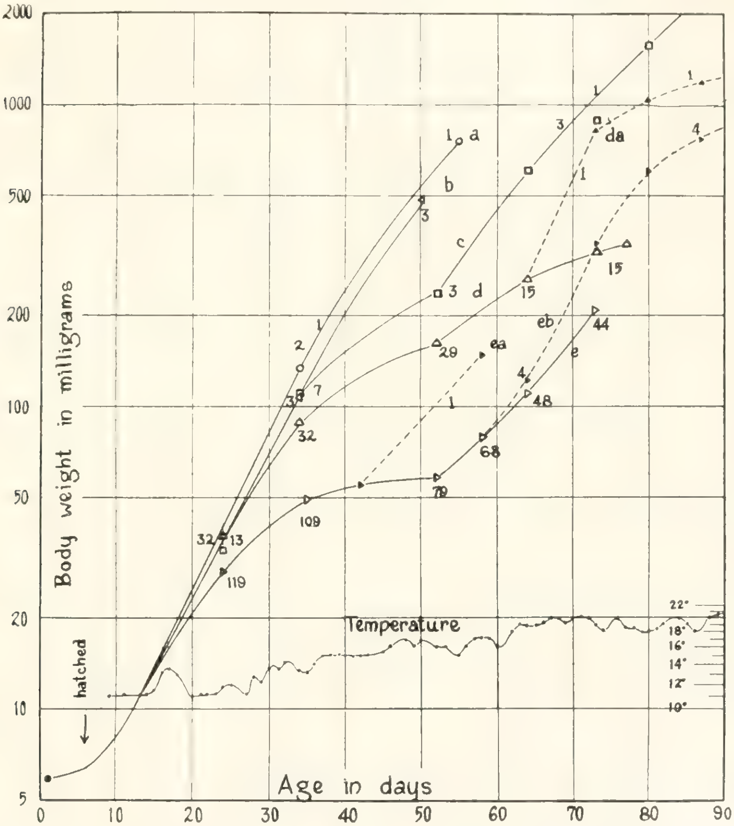

FIG. 12. Growth of tadpoles in flowing water at variable temperatures. The cultures contained the varying numbers of individuals indicated by the numerals. Upon the 42d and 58th days respectively, 1 and 4 individuals were isolated from culture *e*, and upon the 64th day 1 individual was isolated from culture *d*, whereupon rapid growth was resumed by the isolated ones. All cultures were from brood *U* in 1500 cc. of rapidly changing water.

Attempts were made to regulate the temperature of this water by first running it through coils immersed in a regulated water-bath. Sufficient heating capacity and coil capacity were not available at the time, and therefore it was deemed sufficient for the present purpose to allow the temperature to vary alike in all the dishes. The mean temperature was much lower in the day than in the night, both gradually rising from week to week during the spring season.

As the water flowed from the tap into the beaker it was led under the surface of the water already present, either inside the cheesecloth bag or else just outside it. In the former case all the tadpoles eventually perished because nitrogen from the warming water accumulated in their tissues; only in the latter manner could this be avoided. It was proven by watching the convection of colored substances that the water at all times mixed through the cheesecloth partitions.

Quite unexpectedly (at the time), the contrast in body sizes became as large in running as in still water. An experiment is represented in Fig. 12. Nothing could be more convincing than the comparison side-by-side of the tadpoles in adjacent beakers after one or two months' growth.

Animals in running water were compared with those in still water by allowing the temperature to vary alike in both. Those in still water occupied dishes that floated in running water. It was found in practice that the floating dishes were always at slightly higher temperatures than the beakers containing cheesecloth bags. For this reason the tadpoles in the still water had an advantage. The essential point is, however, that still water did not sensibly retard growth, as is shown by the data of Table I.

One other factor that differed for the cultures in running water and those at constant temperature was exposure to light. The constant temperature room was dark except during those hours each day when cultures were being cared for, at which times they were exposed to dim artificial light. The tadpoles in running water were exposed to indirect sunlight throughout every day, and never to artificial light. That light and dark were not significant is attested by the experiments with still water under the temperature influence of flowing water, and by numerous experiments in which all comparable cultures were carried on in stagnant water throughout the season under the conditions of a laboratory room. These cultures showed just as significant effects of crowding as any did; they are illustrated by brood *C* in Fig. 8.

The tests with flowing water demonstrated conclusively that the essential influence of crowding is physical rather than chemical. Somehow the tadpole is retarded in proportion to its mean free path of movement.

10. *Partitioning of Spacc.*—The next step was to measure the growth response when individuals were separated into small compartments. Four tadpoles, each one in a small cheesecloth bag, were compared with two of the same brood together in one large cheesecloth bag. All were subjected to the same running water, with the end results shown in Table I. The separated individuals grew significantly faster than those that were together, though each one had on the average less space to itself.

TABLE I

Comparison of tadpoles grown at the temperature of flowing water under diverse conditions at 45 days after fertilization.

Culture	Number of Individuals	Conditions	Final Mean Weight in Milligrams
		Exposed from 7th to 45th days	
X2n	1	Agitation in 1500 cc., flowing	289
X2q	4	Together in 750 cc., *not* flowing	697
X2r	4	Together in 750 cc., *not* flowing	617
X2y	4	Together in 750 cc., *not* flowing	673
X2s	4	Separated in 1500 cc., flowing	1053
X2t	4	Separated in 1500 cc., flowing	1017
X2u	2	Together in 1500 cc., flowing	814
		*Exposed from 26th * to 45th days*	
X3g	1	Agitated in 1500 cc., flowing	738
X3h	1	Alone in 1500 cc., flowing	1154
X3k	16	Together in 1500 cc., flowing	606
X3i	1	Alone in 100 cc., flowing	575

* The mean weight (*X3*) at the 26th day was 190 milligrams.

This result may seem somewhat inconsistent with any conception of a spatial factor. While it is possible that some other influence, such as temperature or food supply crept in unnoticed, the result appears to be as well substantiated as any others in flowing water. The result does not seem to be inconsistent with the conception of locomotor disturbances as the crucial factor in crowding. It is confirmed by experiments in addition to those of Table I in which single individuals in large bags grew no faster than single individuals in small bags so long as they were in flowing water.

11. *Agitation.*—Any influence of size of the environment as a physical factor must be exerted through some sensory means. A tadpole might become aware of the extent of its environment through vision, touch, or muscle senses. Vision is apparently ineffective, because

crowding is just as influential upon growth in the dark. Probably no other form of distance reception requires serious consideration in connection with growth. Touch would be expected to be effective either through the contact of other moving objects or in the course of the individual's own movements; muscle senses would be informing chiefly during the individual's own movements.

If crowding influences growth through disturbance of the passive individual, such disturbance could be simulated in other ways. Stirring of the medium or knocking about of the individual seemed indicated. Arrangements were made to agitate the tadpoles by putting them in cheesecloth bags that were held expanded and lifting these bags up and down in beakers of water. The lifting was done rhythmically by a "windshield wiper," driven by compressed air at rates of 6 to 12 strokes per minute.

More than a dozen such cultures were set up from time to time; in most instances the animals were killed by too great violence in shaking, by the friction of the cheesecloth, or by crushing from the glass weight in the bag. Young tadpoles were particularly sensitive to mechanical friction. The only cases in which growth could be followed for a significant period of time were with tadpoles that were allowed to grow to 200 milligrams before being shaken. In these cases growth was retarded (Table I).

Hence agitation prevents growth. Whether this is the factor that prevents growth in conditions of crowding, or whether this is simply a new form of violence, can hardly be decided. It will always remain possible that the proper kind and amount of agitation will not inhibit growth. Until such a result is realized experimentally, it is permissible to regard agitation as the same kind of interference as crowding.

12. *Narcosis.*—It was possible that sensitivity to touch could be avoided by anesthesia without interfering wholly with growth. Individuals in crowds were treated with chloretone in such a way that they did not move, except in rare instances, for three days at a time. During these three days their controls, in the same-sized crowds, were starved. Then for three days they were taken out of chloretone and with their controls were fed. Such periods were alternated for several weeks. Of course no growth occurred in the non-feeding periods; but in the intervening three-day periods growth regularly occurred. It was not possible to carry such cultures long enough to obtain significant results; apparently the dosage of chloretone required varied with the ages of the tadpoles, for sooner or later the individuals were killed. Here again a type of experiment that appears on paper as ideal became in practice useless.

13. *Ingestion of Food and Crowding.*—Little observation is required to notice that per individual crowded tadpoles do not eat so much as uncrowded ones. This was remarked by previous observers, and Babák (1906) and Elven (1928) ascertained that the digestive tracts of crowded tadpoles tended to be slightly smaller and shorter, relatively as well as absolutely.

When green algæ are being supplied daily to tadpoles it is noticed that as soon as body sizes have become obviously smaller in crowded cultures, the food consumption diminishes. It can be seen that this is so even if crowded individuals are compared with controls of the same body size rather than with those of the same age. Decreased food consumption is a tangible intermediate factor between growth and crowding; it causes decrease of growth rate and it is caused by crowding.

The mechanism of the crowding effect appears to be, therefore, that agitation or sensory disturbance decreases food ingestion. Hence the effect is in the first instance upon the behavior of the tadpoles; it modifies their responses to food. If crowded tadpoles are further observed, relatively little disturbance is seen; there is sufficient time for every individual to eat plenty of food, the animals are simply idle instead of eating. They resemble children who do not eat but sit idly at table because exciting events are going on, if a crude analogy be allowed. It is permissible to call crowding a psychological factor in growth, so far as is now apparent.

Comment

The effects of crowding have been studied primarily in the tadpoles of *Rana pipiens.* In all chief points the mechanism of crowding has been confirmed in *Rana sylvatica.* In the literature are recorded partial similar results upon *Rana temporaria* (Babák, 1906; Kříženecký and Podhradsky, 1924) and *Rana esculenta* (Yung, 1885; Bilski, 1921), and upon a great number of other kinds of organisms of which the chief is the snail *Lymnea* (see Crabb, 1929). It is impossible to say that these spatial influences are alike in all species; for in other species they have not been fully analysed, and there are some indications (Goetsch, 1924) that they are not alike.

The experimental results on *Rana pipiens* show that chemical effects of crowding are insignificantly small. In rapidly running water the full effects of crowding are demonstrable. They can be simulated by agitation of the tadpoles. Both crowding and agitation were observed to discourage the assimilation of food. The situation is not that food energy is used up for motor responses to touch instead of being retained for growth, but that the food is actually not eaten. There is sufficient time available for eating, but the behavior toward food is modified by agitation.

It would be possible to look upon the decreased ingestion as a measure of conservation, for in crowded conditions food supply will under most natural conditions run short. This behavior has the biological result that little more food than is required for bare maintenance is eaten. Whether this is a behavior of foresight on the part of a tadpole, no one can state.

It may be pointed out that the demonstration, in Fig. 8, that body weight is denied the crowded tadpoles in proportion to the square root of their density ($\sqrt{n}$), agrees with what might be expected upon the conception of disturbance by collisions. The number of random collisions within a unit of time would be proportional to $\sqrt{n}$. This agreement, however, by no means excludes other views.

The occurrence of growth inhibition in nature under conditions of limited extent of environment has been reported by many observers. Almost everyone who watches pond life has seen small undeveloped tadpoles in small ponds upon the same day that all tadpoles of the same species have already metamorphosed and left ponds of greater extent. Of course, many factors differ in these situations, and it is almost certain that no conclusive experiment will occur outside the laboratory.

In non-aquatic organisms effects of crowding have frequently been observed; indeed, they have often been discussed in man. The only species for which effects have been measured under highly controlled conditions is the fly *Drosophila*. In it Pearl (1928) demonstrated effects of crowding upon longevity and upon fertility. He believed that chemical influences had been ruled out and that crowding exerted its effect through the psychological patterns of the flies. It may be added that Pearl's data on fertility fit the formula presented above for the relation between body weight and density of crowding, substituting the number of eggs laid for the weight factor.

Crowded tadpole cultures contain individuals highly diverse in weight. Ordinary observation of this fact is confirmed by numerous measurements of weight, which will not be presented because they have relatively small bearing on the problem of the crowding mechanism. The data were obtained by weighing the smallest and largest individuals in cultures of 32, in cultures of 4, and in duplicate single cultures. Instances where the largest individual weighed five times as much as the smallest were found repeatedly. In an equal number of single cultures belonging to one brood, the largest never differed from the smallest by 20 per cent of the weight on any one day. The variability is remarkably small during growth under optimal conditions; crowding, and probably any other limiting condition, increases it. The problem of variability of size was discussed by Křižinecký and Cetl (1924) in an attempt to relate inequalities of size to " intensity of assimilation." Whether assimilation be a physiochemical or a psychological phenomenon, their correlation remains very indefinite except as they express assimilation in terms of the concentration of food *available* to the tadpoles. In the present experiments it seemed simple to picture the development of inequalities in terms of variable aggressiveness in feeding. Plenty of food was available, but only the type of behavior that was influenced less by crowding would allow the ingestion of much food.

Very probably the smaller sizes of densely crowded tadpoles are accompanied by disproportions of some organs and tissues. Slight evidence has been cited that such is the case for the intestine. The endocrine organs might be suspected of showing deficiencies or hypertrophies. Whether such unusual conditions serve as causal or intermediary factors in the control of body size can only be surmised.

The importance of crowding in any experiment having to do with growth in tadpoles is evident. No conclusions can be drawn from mass cultures unless both the number of individuals and the total weight of the individuals present in each culture are equated daily by discarding appropriate tadpoles each time a death occurs. It must be emphasized that crowding is proportional not only to numbers of individuals, but also to the sizes of the individuals. In this way tadpoles that have grown large will inhibit one another's growth much more than the tadpoles whose growth has previously been inhibited.

Concerning the problem of body size, it may be said that the tissues of animals attain a steady size in the adult not because *they* cannot grow further, but because their environment prevents them from growing. This is attested by the whole body of facts obtained through observation of regenerating tissues and of explanted tissues. Hence it is the inhibition of growth which is interesting, for many tissues appear to have the ability to undergo unlimited logarithmic increase. In tadpoles and other aquatic species it is recognized that not only the body fluids, but also the environing media, limit the rate and amount of growth. This variety of influence has now been quantitatively evaluated. It has been found not to be of a direct physical sort, but is effective because the tadpole is a reacting organism. It is exhibited ultimately in every individual even under optimal conditions. It may be concluded that useful growth, like civilization, consists not in the limitless expression of inherent powers, but in the careful gradation of activity to fit circumstances. No other form of response would be equally liable to attain biological success.

SUMMARY

1. Growth of the tadpole under optimal conditions is very slow between fertilization and hatching, proceeds with logarithmic increase of bulk for about two weeks, and then declines in rate up to the beginning of metamorphosis.

2. The crowding of many individuals together causes little change in the initial rate of logarithmic increase, but brings on the decline in rate much sooner and more severely than in isolated individuals. The same effect results from decrease in the volume of water in which the tadpoles live.

3. Experimental analysis of the mechanism of the crowding effect shows that the composition of the water itself has no significant influence on growth. The full effect of crowding is manifested in rapidly running water, but not when the individuals are partitioned from one another. A similar inhibition of growth results from agitation of the tadpoles.

4. The ingestion of food per individual is much reduced by crowding. The effect is therefore exerted upon the behavior of the tadpole toward food. The effect is precisely graded with respect to the density of crowding, so that it is accurately correlated with the physical size of the environment. It possibly serves as an example of the inhibitions through which growth is ordinarily regulated.

BIBLIOGRAPHY

ADOLPH, E. F., 1929. The Quantitative Effect of Crowding on the Rate of Growth of Tadpoles. *Anat. Rec.*, **44**: 227.

BABÁK, E., 1906. Experimentelle Untersuchungen über die Variabilität der Verdauungsrohre. *Arch. Entw. Mech.*, **21**: 611.

BIALASZEWICZ, K., 1908. Beitrage zur Kenntnis der Wachstumsvorgänge bei Amphibienembryonen. *Bull. Internat. Acad. Sci. Cracovie, Cl. Sci. Math. et Nat.*, Ser. B., 783.

BIALASZEWICZ, K., 1912. Über das Verhalten des osmotischen Druckes während der Entwicklung der Wirbeltierembryonen. Teil I und II. Versuche an Hühner- und Froschembryonen. *Arch. Entw. Mech.*, **34**: 489.

BILSKI, F., 1921. Über den Einfluss des Lebensraumes auf das Wachstum der Kaulquappen. *Pflüger's Arch. ges. Physiol.*, **188**: 254.

CHAMBERS, R., 1908. Einfluss der Eigrösse und der Temperatur auf das Wachstum und die Grosse des Frosches und dessen Zellen. *Arch. mikr. Anat.*, **72**: 607.

CRABB, E. D., 1929. Growth of a Pond Snail, Lymnaea stagnalis appressa, as indicated by Increase in Shell-size. *Biol. Bull.*, **56**: 41.

DAVENPORT, C. B., 1897. The Rôle of Water in Growth. *Proc. Bost. Soc. Nat. Hist.*, **28**: 73-84.

DEMPSTER, W. T., 1930. The Growth of Larvæ of Ambystoma maculatum under Natural Conditions. *Biol. Bull.*, **58**: 182.

DONALDSON, H. H., 1911. On the Regular Seasonal Changes in the Relative Weight of the Central Nervous System of the Leopard Frog. *Jour. Morph.*, **22**: 663.

ELVEN, E., 1928. Fütterungsversuche an Kaulquappen, mit besonderer Berücksichtigung der Babákschen Experimente über die Beeinflussbarkeit der Darmlänge durch verschiedene Nahrung. *Arch. Entw. Mech.*, **113**: 61.

FAURÉ-FREMIET, E., ET J. DRAGOIU, 1923. Le premier cycle de croissance du têtard de "Rana temporaria." *Arch. Int. Physiol.*, **21**: 403.

GALLOWAY, T. W., 1900. Studies on the Cause of the Accelerating Effect of Heat upon Growth. *Am. Nat.*, **34**: 949.

GOETSCH, W., 1924. Lebensraum und Körpergrösse. *Biol. Zentralbl.*, **44**: 529.

HALBAN, J., 1910. Die Grössenzunahme der Eier und Neugeborenen mit dem fortschreitenden Alter der Mutter. *Arch. Entw. Mech.*, **29**: 439.

HOGG, J., 1854. Observations on the Development and Growth of the Water-snail (*Limneus stagnalis*). *Trans. Micr. Soc.* London, N. S., **2**: 91.

KŘIŽENECKÝ, J., 1917. Über das Verhalten lebender Froscheier und Froschlarven in destillierten Wasser. *Arch. Entw. Mech.*, **42**: 604.

KŘIZENECKÝ, J., AND V. CETL, 1924. Über die Abhängigkeit der Variabilität der Körpergrösse von dem Grade der Assimilationsintensität. *Arch. mikr. Anat. Entw. Mech.*, **100**: 164.

KRIŽENECKÝ, J., UND J. PODHRADSKY, 1924. Studien über die Funktion der im Wasser gelösten Nährsubstanzen im Stoffwechsel der Wassertiere. III. Mitteilung. Zur näheren Kenntnis der wachstumssteigernden Wirkung im Wasser gelöster Nährsubstanzen unter normaler Fütterung mit geformter Nahrung. *Arch. ges. Physiol.*, **204**: 25.

MILLER, N., 1909. The American Toad (*Bufo lentiginosus americanus,* Le Conte). A Study in Dynamic Biology. *Am. Nat.*, **43**: 641.

PATCH, E. M., 1927. Biometric Studies upon Development and Growth in Amblystoma punctatum and tigrinum. *Proc. Soc. Exper. Biol. Med.*, **25**: 218.

PEARL, R., 1928. The Rate of Living. New York.

PFLÜGER, E., 1883. Das Ueberwintern der Kaulquappen der Knoblauchkröte. *Arch. ges. Physiol.*, **31**: 134.

MORGAN, T. H., 1906. Experiments with Frog's Eggs. *Biol. Bull.*, **11**: 71.

SCHAPER, A., 1902. Beiträge zur Analyse des thierischen Wachsthums. I. *Arch. Entw. Mech.*, **14**: 307.

SEMPER, C., 1873. Ueber die Wachstums-Bedingungen des *Lymnaeus stagnalis. Arb. Zool.-Zoot. Inst. Würzburg,* **1**: 137.

SPENCER, H., 1866. The Principles of Biology. 2 vols. New York.

SPRINGER, A., 1909. A Study of Growth in the Salamander, *Diemyctylus viridescens. Jour. Exper. Zool.*, **6**: 1.

TERROINE, E. F., ET H. BARTHELEMY, 1921. De l'existence de rapports biométriques entre la Grenouille rousse (*Rana fusca*) et ses oeufs à l'époque de la ponte. *Compt. rend. Acad. Sci.*, **173**: 740.

YUNG, E., 1885. Influence du nombre des individus contenus dans un même vase, et de la forme de ce vase, sur le développment des larves de Grenouille. *Compt. rend. Acad. Sci.*, **101**: 1018.

BODY SIZE AS A FACTOR IN THE METAMORPHOSIS OF TADPOLES

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INTRODUCTION

The rôle of body size in the activities of organisms has been studied only in a comparative way. Its effectiveness can be demonstrated experimentally, however, within a single species in relation to a number of physiological functions. One of these functions in Amphibia is metamorphosis.

When many tadpoles were reared in one aquarium, as is described in the preceding paper, they were retarded markedly in growth as compared with isolated individuals. When the time came for the isolated individuals to metamorphose, certain of the partially crowded individuals were also able to metamorphose. But the body weights of the latter were much less than those of the isolated individuals. Other tadpoles crowded more densely did not metamorphose at this time, but were able to metamorphose at later times. To analyse these relationships, data were obtained under controlled conditions upon the changes of body weight and the ages at which the morphological changes of metamorphosis occurred.

In the preceding paper size was regarded as a result conditioned *inter alia* by crowding. In the present paper size is to be considered as a condition, the result produced being *inter alia* metamorphosis.

Two species, *Rana sylvatica* and *Rana pipiens,* were used in the observations, and the ages at metamorphosis were recorded for about 190 individuals that had been reared under known conditions. In selected instances the body weights of single individuals were measured; in other instances groups of individuals were followed with respect to body weight through metamorphosis. That crowding resulted in delay in tadpole metamorphosis was reported by Yung (1885), but no data on body weight were obtained by him. The present experiments were recently summarized in abstract (Adolph, 1930).

Weight Changes During Metamorphosis

The progressive changes of body weight in tadpoles crowded to varying extents are shown in Fig. 1. Some time after the increase of body weight of the tadpole has fallen below the initial "logarithmic" rate, the percentage increment is greatly reduced, and then increase ceases. Finally body weight is lost rapidly for about two weeks, at the end of which time metamorphosis is visibly complete.

The tadpole has ceased to grow before most of the morphological changes of metamorphosis are apparent. The first changes are the budding of the hindlegs; no others are observable ordinarily until the tadpole has begun to lose weight.

After metamorphosis is complete, the body weight of the frog, if not fed, is almost constant for many days. There is a slight gradual loss due to the fact that body tissue is being used as the source of metabolic materials. Three or four weeks (at 19° C.) after the maximum weight of the tadpole is reached, the final weight of the metamorphosed frog is attained. On the average 60 per cent of the body weight is lost in this four-week period.

The forelegs usually burst forth from under the skin when about one-third of the metamorphic loss of weight has occurred. This event is the most convenient morphological event to identify, and in the present study it has been used as the criterion of metamorphosis. But when thus referred to the changes of body weight it is found that the appearance of the forelegs varies considerably in time of occurrence. If a single criterion of metamorphosis is required, the best one would seem to be the point at which the body weight is halfway between the maximum weight of the tadpole and the weight of the frog three or four weeks (or an equivalent age at any other temperature) thereafter; for the change in body weight represents an average of all the changes that are occurring in the body.

The tremendous loss of weight during metamorphosis does not represent a 60 per cent reduction of all chemical constituents of the body. While it is well known that catabolism of nitrogen and other substances is increased at this time, the losses through catabolism do not nearly correspond to the losses of weight. The percentage of solids is known to increase markedly (Schaper, 1902), and this change alone accounts for a considerable portion of the loss of body weight.

Body Weight and Metamorphosis

At 19° C. the maximum weight attained before metamorphosis in *Rana sylvatica,* brood *Q,* was 1230 mg. and in *Rana pipiens,* brood *U,* was 3425 mg.

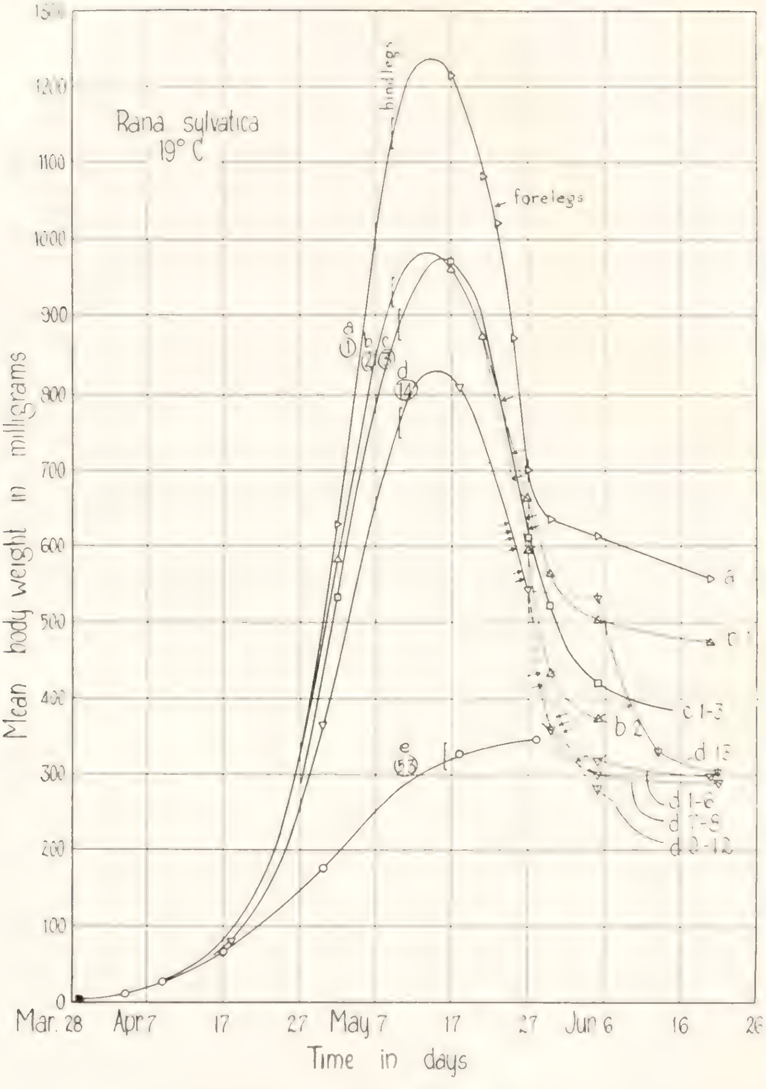

FIG. 1. Growth in weight of five cultures of *Rana sylvatica*, brood *Q*, at 19° C. The numbers of individuals contained in the cultures are indicated in circles; each culture was in 1000 cc. of tap water, having a surface of 550 sq. cm. and a depth of 1.8 cm., that was changed once a week. The first appearance of hindlegs in the culture is indicated by a bracket, and the appearance of forelegs, which was taken as the sign of metamorphosis, is indicated for each individual by an arrow. The body weights after metamorphosis were determined for smaller groups of individuals which were numbered in the order of metamorphosis. The subsequent history of culture *e* is indicated in Fig. 4.

When the uncrowded tadpoles of a brood metamorphosed, the slightly crowded individuals also metamorphosed, and each of the events marking this transformation occurred upon almost exactly the same day for all individuals. But, as Fig. 1 shows, the body weights that had been attained upon the day when the decrease of weight began were diverse. With a density of fourteen individuals per liter the mean weight was only two-thirds of the weight where the density was one per liter.

During metamorphosis the same relative differences of size were maintained, so that the resulting frogs were of diverse sizes. There was, however, a slight tendency for the smaller tadpoles to lose a larger percentage of their body weights in the transformation process. Hence the percentage diversity of sizes was somewhat greater among the complete frogs than among the tadpoles.

The great contrast in the sizes of frogs is illustrated by the frequency curves of maximum weight represented in Fig. 2. The sizes are thus shown to depend primarily upon the amount of growth that was previously allowed by the density of the tadpole population (or other limiting factor).

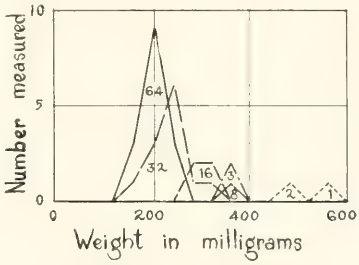

FIG. 2. Frequency distribution of the final weights after metamorphosis in all cultures of *Rana sylvatica,* brood *Q,* at 19° C. The numbers indicate the initial densities of the tadpole populations in individuals per 1000 cc.

In addition to knowing the sizes attained by individuals that metamorphosed, it is important to know the sizes of tadpoles that did not metamorphose. Is there any sharp limit of body weight that determines whether or not metamorphosis shall occur? The maximum weights attained by the largest tadpoles not metamorphosing and the other tadpoles metamorphosing are given for one brood of *Rana pipiens* in Table I. So far as data are available, they indicate that body weight constitutes a decisive quantitative factor in metamorphosis. At 19° C. the upper limit of size that did not allow metamorphosis within 300 days after fertilization was 2200 mg. for brood *U, Rana pipiens* (individuals *ta* and *va*), and within 150 days was about 550 mg. for brood *Q, Rana* *sylvatica;* in the latter brood the tadpoles that did not eventually metamorphose were very few.

AGE AT METAMORPHOSIS

At 19° C. uncrowded individuals of *Rana sylvatica,* brood *Q,* acquired forelegs at the age of 54 days. In *Rana pipiens,* brood *U,* the corresponding stage was attained at 117 days. The tadpoles that were slightly crowded were able to metamorphose at the same time as uncrowded ones. Hence within certain limits the body size had little influence upon the time of onset of metamorphosis. But tadpoles that were densely crowded did not metamorphose at the same age as uncrowded ones. This is apparent in the two densest populations of Fig. 1.

TABLE I

Ages and weights during metamorphosis of individuals of Rana pipiens, brood U, at 19° C.

Designation	Age at Appearance of Forelegs	Body Weights on the 105th Day	Maximum Body Weight	Body Weight at Appearance of Forelegs	Final Body Weight After Metamorphosis
	days	*mg.*	*mg.*	*mg.*	*mg.*
ca	117	2735	2735	1400	—
j	117	3425	3425	2450	—
la	118	2730	2770	2060	—
ua	119	—	—	1510	1300
qaa	120	2880	2890	1860	1360
ub	148	—	2750	—	1110
raa	152	—	2860	—	—
oa	225	—	—	—	—
ta	—	1910	—	—	—
va	—	2000	—	—	—
rab	343	—	—	—	—
qab	344	1585	—	—	—
ma	457	1930	—	—	—

If the frequency of various ages at which metamorphosis occurs is plotted, as in Fig. 3, the contrast is great. The most densely crowded individuals not only never metamorphosed at so young an age as uncrowded ones, but various individuals metamorphosed at highly diverse times.

The diversity of ages at which metamorphosis occurred is illustrated in detail in Fig. 4. Over a period of more than two months transformations frequently occurred in the particular culture illustrated. Of course, the transformations cannot be said to have occurred at random, for in each case it was usually the largest tadpole that began to metamorphose next.

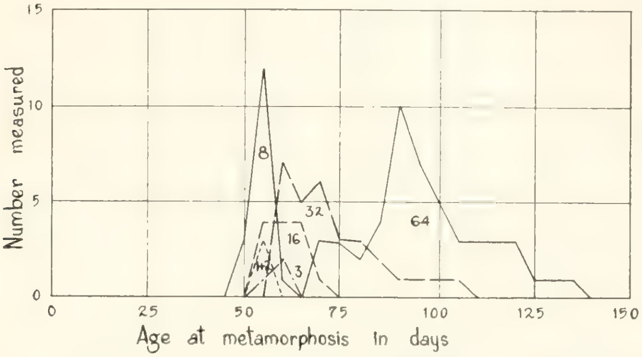

FIG. 3. Frequency distribution of the times (ages) of appearance of forelegs in all cultures of *Rana sylvatica*, brood *Q*, at 19° C. The numbers indicate the initial densities of the tadpole populations.

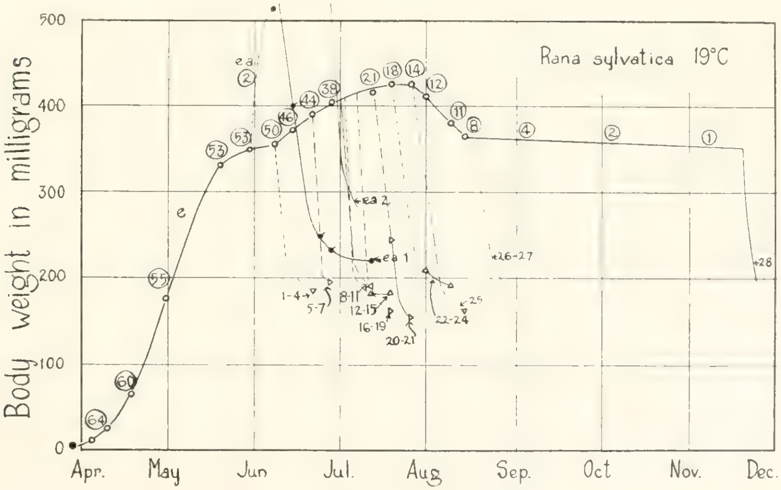

FIG. 4. Sequence of body weights in culture *Qe*. Each few individuals that metamorphosed were weighed after the forelegs had appeared, the individuals being numbered consecutively as they metamorphosed. The total numbers of individuals in the culture are indicated in circles. Two average individuals *ea* were isolated into 1000 cc. before any had metamorphosed; these were able to grow considerably before they transformed.

In the brood shown in Fig. 4, the last individual that had survived came to metamorphose 237 days after its growth started. Its age was then 440 per cent of the age when the uncrowded individuals of the same brood metamorphosed, which may be referred to as "par." In *Rana pipiens* the last survivor metamorphosed at the age of 457 days (Table I), which was 390 per cent of par. While metamorphoses are frequent at ages near par, they become less frequent per unit of the population exposed to metamorphosis as age increases. This is due not to the death rate among the retarded tadpoles, but to the fact that the condition which must be met before metamorphosis can occur, which is body size itself, becomes slower in rate of attainment.

Body Weight and Age

The interaction of the two factors of metamorphosis, namely, size and age, may now be evaluated. It was found, as shown in Table I, that individuals that were just on the verge of attaining the size necessary for metamorphosis were still able to metamorphose after a delay of some weeks or months, even though they made little or no further gain in weight. The charts of body weight indicate that metamorphosis to the extent of stopping growth in weight might be said actually to have occurred at par age, but the morphological changes of metamorphosis did not proceed. Evidently, within certain limits, a deficiency in body size can be compensated by an increase of age.

The way to compare the rôles of the size factor and the age factor in metamorphosis is to plot the two together. This is done in Fig. 5 for the one brood on which most data are available. Since the known body weights are more numerous after the completion of metamorphosis than at the beginning of metamorphosis, the final weight of the frog is used as the measure of body size. The same sort of curve results, however, whether maximum weight of the tadpole or weight on the day that forelegs are acquired be used in place of final weight of the frog.

The best curve drawn empirically through the points of Fig. 5 is a rectangular hyperbola. If W is the body weight in milligrams after the completion of metamorphosis, A is the age at which the forelegs broke through in days after fertilization of the egg, and c, d, and e are constants, the relationship $(A - e)(W - d) = c$ represents the graph. The constants d and e represent the asymptotes of the hyperbola. The conclusion may be drawn that no possible increase of size would allow metamorphosis to occur before e days of age, and neoteny would last indefinitely if sufficient body size to result in a frog of weight d were not attained.

For the brood Q at 19° C., living on the diet of *Spirogyra, Vaucheria,* and spinach used, the minimum age e is 51 days, the minimum body weight d is 160 mg., and the constant c is 1200 day-milligrams.

The curve as drawn in Fig. 5 represents these values. Under the conditions in which brood *Q* was reared, the influence of other factors upon the initiation of metamorphosis was evidently small. Body weight and age were the effective factors in conditioning the onset of metamorphosis.

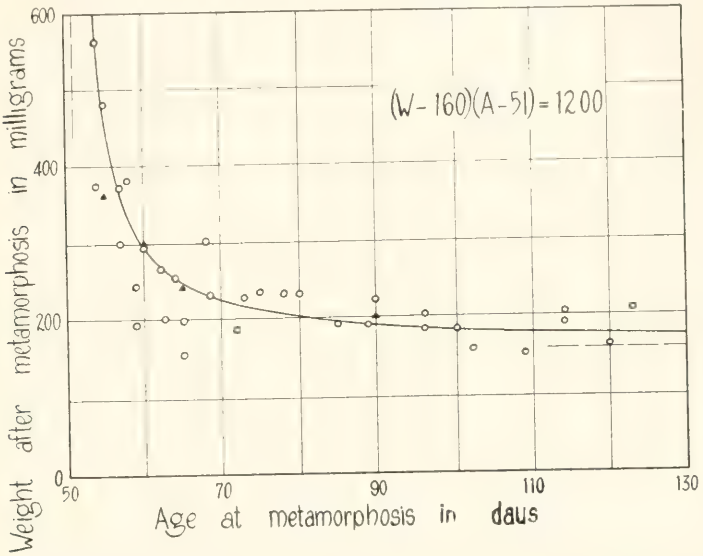

FIG. 5. Correlation between the ages at which the forelegs appeared and the weights of the frogs after metamorphosis was complete, in all individuals of *Rana sylvatica*, brood *Q*, at 19° C. The curve drawn through the points is represented by the formula for a hyperbola, the dashed lines being the asymptotes. The four solid points represent the modes for various density groups read off from Figs. 2 and 3.

COMMENT

The factors that have been held to be responsible for initiating metamorphosis in Amphibia may be roughly classified as: Age, size, previous history, food, oxygen supply, temperature, hormonal relations, and heredity. The rôles of age and size have been evaluated above by using observations in which the other factors were held largely constant. If under previous history be included rate of growth and crowding, then it has been shown that these are of importance chiefly because they influence size.

Types of food have not been varied in these experiments; and it may be that all the observations reported by others in which the food was varied really influenced metamorphosis either through size or through endocrine mechanisms. Starvation was reported by Barfurth (1887) to initiate metamorphosis in frogs. If his data are analyzed, however, it is found that by no objective test of significance were his starved individuals different from his fed individuals. Powers (1903) concluded from careful observations that sudden starvation precipitated metamorphosis in *Amblystoma*. Several attempts were made during the present experiments to bring on metamorphosis by denying food to tadpoles that had almost attained the minimum size required for metamorphosis. But none metamorphosed without further feeding.

High oxygen tensions were stated by Huxley (1925) to inhibit the metamorphosis of frogs. Extirpation of the lung rudiments by Helff (1931) had no significant effect upon the time of metamorphosis. The necessity of rising to the surface for air, the amount of gill surface, and the contact with air are said by Powers (1903) to be of no consequence in the metamorphosis of *Amblystoma*.

The temperature was held constant in the present experiments. Uhlenhuth (1919, 1921) reported that when grown at low temperatures, certain urodeles not merely took longer to attain metamorphosis, but grew to a larger size before metamorphosis.

The influence of heredity has never been studied in frogs apart from environmental factors. That broods differ within the same species is possibly indicated by the varying reports of size at metamorphosis. Thus, in *Rana pipiens* Kuntz (1924) reported that the maximum size of the tadpoles was 6.8 grams, while Helff (1926) reported that the maximum size was 1.4 grams. In both cases, nevertheless, 57 per cent of the body weight was lost before metamorphosis was complete.

The evaluation of size as a factor in determining the onset of metamorphosis does not imply that size is an independent variable. When all the factors are quantitatively known, it will probably be found that most of them are both effects and causes. It may be that one or another chemical or physical condition will appear to set aside the usual complex of conditions. It is already known that thyroid feeding will render the size factor unnecessary for metamorphosis; very small and young tadpoles thereby attain adult properties. But the rate of an endocrine activity is coördinately correlated with many other factors, and it would be almost anomalous if it ultimately proved true that a single limiting factor ordinarily controlled the onset of metamorphosis.

For anyone who desires to visualize a causal concatenation of factors, a schema to which the author does not subscribe, a possible mechanism by which size and metamorphosis are correlated may be pictured as follows. It is well known that metamorphosis is often controlled by the activity of the thyroid gland. This gland is ordinarily thrown into sufficient activity to induce metamorphosis only in the presence of anterior pituitary tissue from metamorphosing tadpoles (Smith, 1923;

Uhlenhuth, 1928; Ingram, 1929). But the anterior pituitary also often controls the rate of growth, and hence the body size (Smith, 1918; Allen, 1920). The anterior pituitary must ordinarily attain its ability to set off metamorphosis through its developmental age, but in addition cannot actually set off metamorphosis unless it and other organs have attained a certain size through growth. The failure to attain this minimal size or activity, either absolute or relative, may be due not merely to insufficient nutriment, but equally well to any other influences retarding growth.

Many important physiological reasons may be postulated as to why a tadpole much smaller than the usual should not metamorphose. It is doubtful whether the relative objective importance of any of these reasons could be evaluated. In nature all sizes from a few milligrams to many grams, and all ages from a few days to several years are found to be sufficient for metamorphosis in one amphibian species or another. Almost no physiological block to metamorphosis cannot, it may be supposed, be overcome in the course of evolution. Only the situation as found in particular species can be described as a fit part of the pattern of existence.

Summary

1. Tadpoles of *Rana sylvatica* and *R. pipiens* if sufficiently retarded in growth by crowding did not metamorphose at the same ages as uncrowded ones. They were able to metamorphose at their small sizes at later times. Those only slightly retarded were able to metamorphose at the usual time, becoming small frogs.

2. Within certain limits a deficiency of body weight is compensated by a surplus of age, and a correlation of the two factors has been established. Through retardation of growth in size the larval stage can be greatly prolonged. Body size is therefore a tangible quantitative factor in the complex of conditions which regulate the onset of metamorphosis.

BIBLIOGRAPHY

Adolph, E. F., 1930. Body Size as a Factor in the Metamorphosis of Frogs. *Anat. Rec.*, **47**: 304.

Allen, B. M., 1920. Experiments in the Transplantation of the Hypophysis of Adult Rana pipiens to Tadpoles. *Science*, **52**: 274.

Barfurth, D., 1887. Versuche über die Verwandlung der Froschlarven. *Arch. f. mikr. Anat.*, **29**: 1.

Helff, O. M., 1926. Studies on Amphibian Metamorphosis. II. The oxygen consumption of tadpoles undergoing precocious metamorphosis following treatment with thyroid and di-iodotyrosine. *Jour. Exper. Zool.*, **45**: 69.

Helff, O. M., 1931. Studies on Amphibian Metamorphosis. VI. The effect of lung extirpation on life, oxygen consumption, and metamorphosis of Rana pipiens larvæ. *Jour. Exper. Zool.*, **59**: 167.

Huxley, J. S., 1925. Studies on Amphibian Metamorphosis.—II. *Proc. Roy. Soc.*, Ser. B, **98**: 113.

Ingram, W. R., 1929. Studies of Amphibian Neoteny. II. The interrelation of thyroid and pituitary in the metamorphosis of neotenic anurans. *Jour. Exper. Zool.*, 53: 387.

Kuntz, A., 1924. Anatomical and Physiological Changes in the Digestive System during Metamorphosis in Rana pipiens and Amblystoma tigrinum. *Jour. Morph.*, 38: 581.

Powers, J. H., 1903. The Causes of Acceleration and Retardation in the Metamorphosis of Amblystoma tigrinum: a Preliminary Report. *Am. Nat.*, **37**: 385.

Schaper, A., 1902. Beiträge zur Analyse des thierischen Wachsthums. I. *Arch. Entw. Mech.*, **14**: 307.

Smith, P. E., 1918. The Growth of Normal and Hypophysectomized Tadpoles as Influenced by Endocrine Diets. *Univ. Cal. Publ. Physiol.*, **5**: 11.

Smith, P. E., and I. P. Smith, 1923. The Function of the Lobes of the Hypophysis as Indicated by Replacement Therapy with Different Portions of the Ox Gland. *Endocrinol.*, **7**: 579.

Uhlenhuth, E., 1919. Relation between Thyroid Gland, Metamorphosis, and Growth. *Jour. Gen. Physiol.*, **1**: 473.

Uhlenhuth, E., 1919. Relation between Metamorphosis and other Developmental Phenomena in Amphibians. *Jour. Gen. Physiol.*, **1**: 525.

Uhlenhuth, E., 1921. The Internal Secretions in Growth and Development of Amphibians. *Am. Nat.*, **55**: 193.

Uhlenhuth, E., and S. Schwartzbach, 1928. Anterior Lobe Substance, the Thyroid Stimulator. *Proc. Soc. Exper. Biol. Med.*, **26**: 149.

Yung, E., 1885. De l'influence des variations du milieu physico-chimique sur le développement des animaux. *Arch. sci. phys. nat.* (Genève), Ser. 3, **14**: 502.

STUDIES ON THE PHYSIOLOGY OF THE EUGLENOID FLAGELLATES

III. The Effect of Hydrogen Ion Concentration on the Growth of Euglena gracilis Klebs[1]

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Introduction

Our knowledge of the effect of hydrogen ion concentration on the growth of the euglenoid flagellates is extremely scanty. Only a few organisms have been studied from this viewpoint, and in most cases the results are at best insufficient evidence for definite conclusions. Practically all the observations of this character have been limited to *Euglena gracilis*, except for those of Linsbauer (1915) and Turner (1917) on unidentified species and for the comparative studies of Kostir (1921), Mainx (1924, 1928), and Dusi (1930). The particular problem of the effect of hydrogen ion concentration on the growth of *Euglena gracilis* is one that has received considerable attention for several reasons. The organism is rather unique in that it possesses a very high resistance to acid solutions, and the literature on the subject is most confusing due to its contradictory character and to the fact that the results of most of the writers were obtained by neither accurate nor comparable methods. In most cases the actual hydrogen ion concentration was not determined, in some cases organic acids were used, in other instances the cultures were not bacteriologically pure, and in no case were quantitative methods employed. Therefore it was believed that an investigation, in which these factors of unknown importance were controlled, might prove useful in the development of culture methods and in the further study of the organism; for this reason the present study was undertaken.

This investigation was performed under the direction of Professor R. P. Hall, whom the writer wishes to thank for his advice during the course of the experiments and for his aid in the preparation of the manuscript.

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[1] This paper, together with Parts I and II of this series of studies, was submitted to the Graduate School of New York University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, April 1, 1931.

HISTORICAL SURVEY

The unusually high resistance of *Euglena gracilis* to acid solutions was first recorded by Zumstein (1900), who used citric acid in his cultures in order to reduce the growth of bacteria. He found that *E. gracilis* grew well when 1–2 per cent citric acid was added to the 'earth infusion' used as a medium. Likewise, he obtained very good cultures with .5 per cent peptone to which he had added as much as 4 per cent citric acid. However, he obtained only poor cultures with .5–1.0 per cent tartaric acid and no growth at all with .2 per cent oxalic acid.

Ternetz (1912) repeated the experiments of Zumstein and found that citric acid was non-toxic to *E. gracilis* in peptone, beef-extract, and earth infusions, whereas it was quite toxic in synthetic inorganic media. Furthermore, she was able to detect no difference in toxicity between lactic, tartaric, malic, and citric acids when present in equimolar concentrations.

Pringsheim (1912) performed the same type of experiments but failed to corroborate the findings of Zumstein. He found that when .5 per cent citric acid was added to the peptone medium, *Euglena gracilis* grew very poorly, but he was able to obtain very good cultures with .12 per cent or less citric acid in the same type of medium. Therefore he concluded that the high acid resistance reported by Zumstein was erroneous, and citric acid was non-toxic only in very high dilution. Linsbauer (1915), working with an unidentified species which Mainx (1928) believed was *E. Klebsii*, found that citric acid was certainly toxic in concentrations as low as .07 per cent. Turner (1917), using an unidentified species of *Euglena* in bacterized cultures, found that an alkaline medium was favorable for growth of the organism.

Kostir (1921) made a study of the comparative resistance of seven euglenoids to various concentrations of citric acid. He found that *Euglena gracilis* was far more resistant than the other species used. The order in decreasing magnitude of the resistance of the species studied was: *E. gracilis*, *Phacus anacoelus*, *E. oxyuris*, *E. ehrenbergii*, *E. geniculata* (?), *E. acus*, *E. deses*.

Tannreuther (1923) found that his most healthy cultures of *E. gracilis* were strongly alkaline and that the poorest cultures were either acid or very slightly alkaline. Since his cultures were not bacteria-free, however, these results might have been due to factors other than hydrogen ion concentration.

The next study of the effect of hydrogen ion concentration on *Euglena gracilis* was that of Mainx (1924, 1928), who used bacteria-free cultures in a medium composed of inorganic salts and .25 per cent beef-extract. He found that the organisms grew very well in this medium if it were neutral. He also obtained good cultures when citric acid was added to a final concentration of 1/400 normal, and fair cultures in media containing 1/100 normal citric acid. Furthermore, he obtained very poor growth in cultures containing 1/500 normal NaOH, and no growth in cultures containing 1/100 normal NaOH.

Dusi (1930), using bacteria-free cultures of *Euglena gracilis* in a medium composed of inorganic salts and beef peptone, performed a more complete series of experiments. The possible effects of organic acids were eliminated by using only HCl and NaOH to bring the medium to the desired pH value. The medium was prepared at six different hydrogen ion concentrations, the most acid tubes having a pH value of 3.5–4.0 and the most alkaline ones a pH value of 8.5–9.0. He found that the maximum density of the cultures was approximately the same in media with pH values from 4.5 to 8.5, but that the maximum density was attained sooner in the alkaline cultures. He accredits this to a higher rate of division in these cultures. In a later paper Dusi (1930*a*) has reported similar experiments with five other species of Euglena, namely, *E. pisciformis*, *E. stellata*, *E. anabaena* var. *minor*, *E. deses*, *E. Klebsii*.

At the time the present study was undertaken the question of the effect of hydrogen ion concentration on *Euglena gracilis* was highly controversial. The methods and results of Dusi (1930) seem much more accurate than those of previous workers, but even his results were at best qualitative and by no means quantitative. Therefore it was deemed advisable to perform quantitative experiments in an effort to determine the relationship existing between hydrogen ion concentration and the growth rate of *Euglena gracilis*.

Material and Methods

The bacteria-free strain of *Euglena gracilis* used in this series of experiments was obtained from the cultures of the Pflanzenphysiologisches Institut of the German University at Prague through the courtesy of Professor E. G. Pringsheim. Fortunately, *Euglena gracilis* was much better adapted for experiments of this type than most of the other available species, because of its more rapid rate of growth in bacteria-free cultures under known conditions.

The organisms were cultured in 16 × 150 mm. Pyrex tubes plugged with cotton. The tubes were maintained at a temperature of 28.30 ± .05° C. by partial immersion in a water bath designed to accommodate a battery of six 100-watt light globes eighteen inches above the water level. The culture tubes were inclined on a wire rack at an angle of 45° in order that the plugs would not block the path of the light.

The medium adopted for the series of experiments was as follows:

KNO_3	.50 gram
KH_2PO_4	.50 gram
$MgSO_4$	.25 gram
NaCl	.10 gram
$FeCl_3$	.05 gram
Partially hydrolyzed casein	5.00 gram
Distilled water	1000.00 cc.

This medium was formulated and selected because the nature and the relative proportion of the constituents do not change considerably with titration or with autoclaving, such as is the case with media containing ammonium or bicarbonate compounds, which are unstable in alkaline solutions, or calcium sulphate and phosphate, which are only slightly soluble in neutral or alkaline solutions. Furthermore, the medium is well buffered against changes in hydrogen ion concentration within the range in which it was used. *Euglena gracilis* may live in such a medium at pH 6.7 for four weeks without producing a pH change definitely detectable with brom thymol blue. The medium was made up in large quantities and then subdivided and placed in 500 cc. flasks. The medium in each flask was brought to the desired pH value by the addition of normal NaOH or normal HCl. The flasks were then plugged and autoclaved. Equal amounts (always 10 cc.) of the medium were then measured directly into the test tubes by means of a Schelbach side-arm burette graduated to .1 cc. The tubes were plugged with cotton and autoclaved and were kept in a cool place until used.

Stock cultures for inoculation were grown in 250 cc. Erlenmeyer flasks in the above medium at a pH of 7.0. Transfers were made from rapidly dividing stock cultures of 10 to 14 days of age in which practically all the organisms were in the flagellated condition. Inoculations were made by means of sterile 12-inch Mohr measuring pipettes of 1 cc. capacity. The stock culture was shaken for five minutes before inoculations were begun and was then reshaken before each inoculation. The usual bacteriological method of aseptic transfer was used.

Measurements of hydrogen ion concentration were made with a La Motte comparator. The pH value was determined after inoculation for one sample tube of each set, and the pH values were determined for all other tubes at the end of the experiment. Readings were, in general, accurate to one-tenth of a pH unit, and the final values never varied more than this amount from the initial pH value except where otherwise stated (Series III*a* and IV*a*).

The ability of the organisms to grow at various hydrogen ion concentrations was measured by comparing the initial concentration of organisms with the concentration in each tube at the end of a definite time. The same method described in Part I (Jahn, 1929) of this series of studies was used for counting the flagellates. In all cases the number of organisms was counted in at least fifty cubic millimeters of each sample, and three samples were counted from each tube. In all cases the concentrations of at least two and usually three tubes were averaged in order to determine the position of each point on the concentration-pH curve.

Experimental Results

Four series of experiments were performed, and each series will be described in detail.

Series I

This series was of a preliminary nature. The medium used was the same as that described above, with the exception that the partially hydrolyzed casein used was composed of one sample of Difco Tryptophane Broth. The stock solution was brought to pH 2.0 by the addition of normal HCl, and then each flask was brought to the desired pH value by the addition of normal NaOH. The pH values of the medium after autoclaving ranged from 3.6 to 8.9. After inoculation at the beginning of the experiment the range was only from pH 3.9 to 8.3, due to the neutralizing effect of the 1 cc. of a rich stock culture in the same kind of medium at pH 6.7 which was used as an inoculum in each case. Four tubes were inoculated at each pH value to be tested, and one tube at each pH value was chosen at random and tested colorimetrically to determine the initial pH after inoculation. Three extra tubes at pH 7.0 were inoculated so that they could be used to determine the initial concentration for the series. The average initial count for the three tubes was .9 thousand per cc., and this was considered to be the initial count for every tube of the series.

At the end of five days the concentration in one tube of each pH value was determined. The concentrations in every case were between 5.7 and 6.4 thousands per cc., and, considering the fact that only one tube of each set was counted, this variation is within the experimental error and can not be considered further. It was decided to count the other tubes at a later time when differences, if present, would be more pronounced. The second count was made on the twelfth day after inoculation. The results are shown in Fig. 1. The curve shows two maxima, one at pH 3.9 and one at pH 6.8, and two minima, one at pH 5.5 and one at the highest pH value used, 8.3.

This bimaximal curve was unexpected, and an explanation was not immediately evident. However, since a trypsin-like enzyme had been reported for *Euglena gracilis* (Mainx, 1928), and since the optimum pH for the digestion of casein may be between pH 6.0 and pH 7.0, it was presumed that the higher growth rate in this range could be explained on the basis of more available necessary food material derived by more complete digestion of the casein. However, this point was not proven, and the high growth rate at pH 3.9 was yet unexplained. It was thought possible that acid hydrolysis of the casein decomposition products might have led to the presence of a higher concentration of available food material in this more acid range. With this in view, a series of amino-nitrogen determinations were performed on unused portions of the medium made up at the same time as that used in the experiments.

The formol titration method of Sorensen was used to determine the relative amounts of amino-nitrogen present in the samples. Four determinations were made for each flask of medium tested, and in all cases 7.0 ± .3 cc. of N/100 NaOH was necessary to restore the pink color to the solution. These results, of course, showed no significant differences in amino-nitrogen content of the media at different pH values. However, only a slight hydrolysis might have given rise to decomposition products of very high growth-accelerating power, and a slight hydrolysis could hardly be detected by the method used.

Series II

This series was started before the final results were obtained from Series I, and it is in some respects a repetition of the former. However, the results are quite different. The initial concentration of the organisms after inoculation was 1.8, and the range was from pH 3.9 to pH 7.9. The final concentrations were determined at the end of ten days. The results are shown in Fig. 1. It is seen that the maximal growth occurred in the most acid tubes. The minimum present at pH 5.5 in the previous series has apparently shifted to 6.5, and the more alkaline minimum of the previous series has failed to make its appearance. It was believed that the minimum present in the acid range in these two series was due to the lack of some particular decomposition product present in the more acid and in the neutral and alkaline ranges. Therefore, it was decided to provide the organisms with a more varied mixture of casein decomposition products, to make the initial concentrations very low, and to make the final counts before the organisms became numerous enough to exhaust any one type of food material. Such experiments are described as Series III and IV.

Series III

The method used in this series was the same as that employed in the two preceding ones. The medium was composed of the same inorganic compounds, but the partially hydrolyzed casein consisted of material from three different samples of Difco Tryptophane Broth, one of which was lighter in color and much more readily soluble than the other two, and of two samples of Difco Tryptophane Broth which had been subjected to peptic and tryptic digestion. One of these had been digested by pepsin for two days and by trypsin for two days; the other had been digested by pepsin for two days and by trypsin for four days. These two mixtures and the three samples of Difco Tryptophane Broth were mixed in approximately equal amounts. The stock solution of the medium was made up at pH 7.0, and then each sample was titrated to the desired pH value by the addition of normal HCl or normal NaOH.

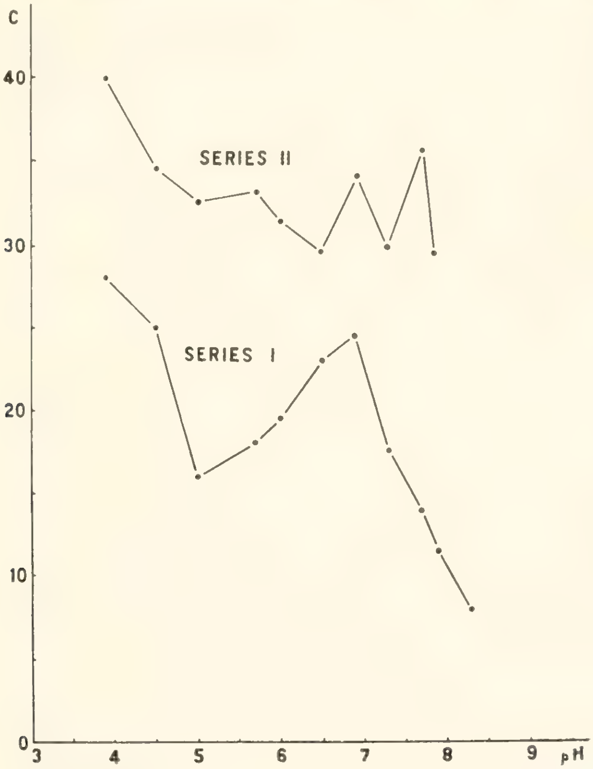

FIG. 1. Graph showing the results of Series I and II. The concentration of organisms in thousands per cc. (*C*) is plotted against pH. Each point represents the average of the concentrations of organisms in two or three tubes of the same pH value.

The initial concentration after inoculation was .1 thousand per cc., and the pH range was 2.0 to 9.9. The concentrations of organisms were determined at the end of nine days. The data obtained are shown in Fig. 2. It is apparent that the organisms grew more rapidly between pH 4.0 and pH 7.5 than in the more alkaline range. The optimum at pH 6.6 is still explainable as being due to the presence of a trypsin-like enzyme with an optimum at pH 6.7 or thereabouts. It is also evident that no growth took place between pH 2.0 and pH 3.6, and that little growth occurred at pH 9.9.

Series IV

This series was, in all essentials, a repetition of Series III. The initial concentration was .85, the pH range after inoculation was 2.0 to 9.9, and the concentrations of organisms was determined at the end of eight days. The results obtained are very similar to those of Series III, and they are also given in Fig. 2. The optimum at pH 6.6 will bear the same interpretation as that given above. Since the pH values of the tubes of Series IV correspond exactly to those of Series III, corresponding values were averaged, and the results were plotted as Curve V of Fig. 2. The curve shows a decidedly greater amount of growth between pH 4.1 and pH 7.5 than in the more alkaline range. The opti-

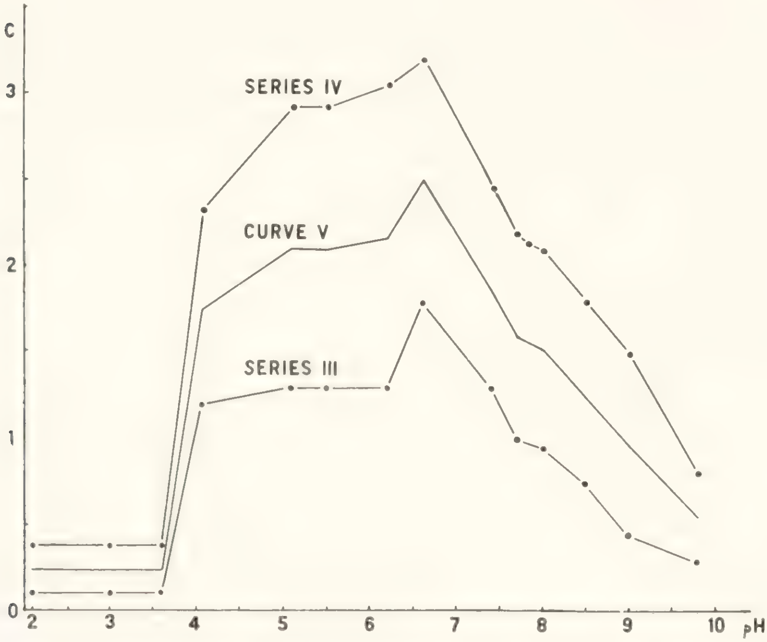

FIG. 2. Graph showing the results of Series III and IV. The concentration of organisms in thousands per cc. (*C*) is plotted against pH. Each point represents the average of the concentrations of organisms in two or three tubes at the same pH value. Curve V was obtained by averaging corresponding values of Series III and IV.

mum is at pH 6.6 and was probably due to the presence of more available necessary food material produced by the action of the trypsin-like enzyme.

Series IIIa and IVa

In each case the concentrations of the organisms in only two or three of the four tubes inoculated at each pH value in Series III and IV were determined in order to obtain the curves shown in Fig. 2. The other one or two tubes of each set were allowed to remain undisturbed and were examined at the end of seven weeks. At this time some of the organisms were encysted on the sides and bottoms of the tubes, and accurate counts were almost impossible. However, the results of macroscopic examination and of pH determinations are shown in Table I. Practically the same results were obtained in both series, and the two are summarized in the table. These results will henceforth be referred to as those of Series III*a* and IV*a* in order to distinguish them from the quantitative results obtained in Series III and IV at the end of 8–10 days.

TABLE I

Initial pH	Amount of Growth	Encystment	Final pH
2.0	—	—	2.0
3.0	—	—	3.0
3.6	—	—	3.6
4.1	+	slight	4.2
5.2	++	slight	5.2
5.6	++	moderate	5.8
6.6	+++	moderate	6.8
7.5	+++	moderate	7.2
7.7	++++	very slight	7.4
8.0	+++	moderate	7.6
8.5	++	moderate	7.8
9.0	+	moderate	8.5
9.5	±	none	9.5

Key to the amount of growth:

—	none.
±	very slight.
+	slight.
++	moderate.
+++	abundant.
++++	very abundant.

Tests for a Proteolytic Enzyme

In order to confirm the existence of a proteolytic enzyme which might account for the optimal amount of growth at pH 6.6, inoculations were made into gelatin and into litmus milk media. Observations at the end of four weeks showed doubtful liquefaction of gelatin and no appreciable effect on litmus milk. However, at the end of twelve weeks the gelatin cultures were almost completely liquefied, and considerable peptonization of milk and reduction of litmus were quite evident. These results confirmed the existence of a proteolytic enzyme as reported by Mainx (1928).

Discussion

At the time the present study was undertaken, the question of the relation of hydrogen ion concentration to the growth of *Euglena gracilis* was a highly controversial one. The results obtained by previous investigators were in a number of cases directly contradictory. The results of Zumstein (1900), Ternetz (1912), Pringsheim (1912), Kostir (1921), Tannreuther (1923), and Mainx (1924, 1928), although indicative of the effect of hydrogen ion concentration on *E. gracilis*, were complicated by at least one other factor such as the use of organic acids, inaccurate measurements of hydrogen ion concentration, or lack of bacteria-free cultures. The results of Dusi (1930) are not invalidated by such factors, but the observations were qualitative only and, as such, are not very informative as regards the effect of hydrogen ion concentration on division rate. The present investigation is an attempt to determine in a quantitative manner the relationship existing between the rate of multiplication of *Euglena gracilis* and the hydrogen ion concentration of the medium.

The curves presented in Fig. 2 may be taken as a measure of the ability of motile stages of *E. gracilis* to grow in a medium composed of certain inorganic salts and casein decomposition products at different pH values, and the curves of the two series of experiments seem to check as closely as might be expected. The maximum at pH 6.6 is probably due to the presence of a tryptic-like enzyme which exerts an optimum action on casein at pH 6.7. The presence of a proteolytic enzyme in cultures of *E. gracilis* has been demonstrated by Mainx (1928), and its existence is confirmed by the gelatin liquefaction and milk peptonization experiments of the author. The gradual decrease in the amount of growth with increasing alkalinity as shown in the curves from pH 6.6 to pH 9.9 checks very closely in both series and is quite the type of decrease that might be expected. The sharp rise from pH 3.6 to pH 4.1 might possibly be criticized if the range were not so great, but inasmuch as the range extended from pH 2.0 to pH 9.9, it was not practicable to use pH intervals smaller than those presented. However, such a sharp rise in growth-pH curves has been found in the case of acid-resistant bacteria. A similar sharp rise has been demonstrated for *Escherichia coli* and *Bacterium aërogenes* (Cohen and Clark, 1919), and therefore it is not surprising that there should be such a phenomenon in highly acid-resistant protozoa such as *Euglena gracilis*.

The differences between the results of Series III and IV at the end of 8–10 days and at the end of seven weeks (Series III*a* and IV*a*) show that although the flagellates multiplied much more rapidly in the acid and neutral media for a short time after inoculation, the maximal density of population obtained after seven weeks was in tubes of pH 7.4–7.7. Inasmuch as Series III and III*a* were started at the same time from the same stock culture with the same initial inoculation and were maintained under the same conditions, and since the only difference between them is in the length of time the organisms were allowed to multiply, the shift in the optimum amount of growth from acid to alkaline media can not possibly be due to an experimental error. This is also true of Series IV and IV*a*, and the results of Series III and IV and of III*a* and IV*a* check very closely. This shift in the maximal amount of growth is very definite and very consistent in both pairs of experiments.

The results of the present investigation are not in direct contradiction to any of the results of previous workers. However, the fact that the division rate of *Euglena gracilis* is initially higher in acid cultures and that the maximum amount of growth is attained in the alkaline cultures is very useful in attempting to explain the contradictory and apparently valid results of previous investigators. The only disagreement between the present results and those of previous workers is with the results of Dusi (1930), who found that cultures of approximately the same density (macroscopic appearance) were obtained from pH 4.5 to pH 8.5 in a medium composed of beef peptone and inorganic salts. However, this might be due to differences in the time of observation in the two experiments, or perhaps to differences in the medium used.

The reason for such a shift in the maximal amount of growth with time is a matter of conjecture. One theory which may be presented is that there was some unknown limiting factor which inhibited growth in the acid cultures after the first few weeks. However, the possible nature of such a factor is totally unknown. Another theory which might be suggested is that the organisms inoculated into the acid solutions were temporarily stimulated to more rapid growth by the acid and that this stimulus failed to call forth a response after the first few divisions. However, the possible existence of such a growth-stimulating power of acid has not been demonstrated, and may not be disclosed by future investigation. Another theory is that certain hydrogen ion concentrations might induce temporary encystment with a concomitant change in division rate. It has previously been observed that organisms transferred from a neutral medium to a strongly acid one may experience what has been termed an "inoculation shock" and may undergo encystment (Mainx, 1928). However, it seems likely that encystment would induce a temporary decrease in division rate, and therefore, this theory does not seem to be a likely explanation of the present phenomenon. If temporary encystment were accompanied by a temporary increase in division rate, the above results might be explained. Since practically nothing is known about the relationship which probably exists between encystment and hydrogen ion concentration and between encystment and division rate, and since encysted forms were not seen in appreciable numbers in Series III and IV, the importance of these factors in determining the above shift in maximal population can not be stated at this time.

The present results indicate that great care should be taken to determine the time relationships in experiments whose primary purpose is to determine the relationship existing between growth and hydrogen ion concentration. This is necessary in order that the early growth rate-pH relationships as shown in Series I, III, and IV are not overshadowed by other factors which become noticeably effective during a later period in the life of the culture, and which might give rise to later contradictory results such as shown in Series III*a* and IV*a*. It is not clear which of the two pairs of experiments represents the truer approximation to the usual growth rate-pH relationship existing in *Euglena gracilis*. The maximal growth in acid solutions as shown in Series III and IV might be explained as being due to a temporary growth stimulus exerted by the acid, and the maximal growth in alkaline solutions in Series III*a* and IV*a* as being due to limiting factors which prevented continued growth in the acid range. However, since it is somewhat unlikely that a growth-stimulating power of acid, if such exists, would show such a strong influence at the end of ten days, it seems more probable that Series III and IV are truer approximations of the usual growth rate-pH relationship.

SUMMARY

1. The amount of growth of *Euglena gracilis* in cultures of different pH values has been measured quantitatively at the end of 8–10 days and has been estimated macroscopically at the end of seven weeks.

2. It is demonstrated that bacteria-free cultures of *Euglena gracilis*, in a solution of casein decomposition products and under conditions which allow mixotrophic nutrition, show, at the end of 8–10 days, a high growth rate between pH 3.9 and pH 7.5 with a maximum at about pH

6.6, and a uniformly decreasing growth rate with increasing alkalinity between pH 7.5 and 9.9.

3. It is also demonstrated that at the end of seven weeks the most growth is found to have occurred in the alkaline range, and that the maximal density of population is at about pH 7.5.

4. It is shown that the results of previous investigators, heretofore considered contradictory, may be explained on a basis of the time relationships involved.

5. The existence of a proteolytic enzyme in cultures of *E. gracilis* is confirmed.

LITERATURE CITED

Cohen, B., and W. M. Clark, 1919. The Growth of Certain Bacteria in Media of Different Hydrogen Ion Concentrations. *Jour. Bacteriol.*, **4**: 409.

Dusi, Hisatake, 1930. Les limites de la concentration en ions H pour la culture d'Euglena gracilis, Klebs. *Compt. rend. Soc. Biol.*, **103**: 1184.

Dusi, Hisatake, 1930*a*. Limites de la concentration en ions H pour la culture de quelques Euglènes. *Compt. rend. Soc. Biol.*, **104**: 734.

Jahn, Theo. L., 1929. Studies on the Physiology of the Euglenoid Flagellates. I. The relation of the density of population to the growth rate of Euglena. *Biol. Bull.*, **57**: 81.

Kostir, W. J., 1921. The Comparative Resistance of Different Species of Euglenidæ to Citric Acid. *Ohio Jour. Sci.*, **21**: 267.

Linsbauer, K., 1915. Notiz über die Säureempfindlichkeit der Euglenen. *Osterr. bot. Zeitschr.*, **65**: 12.

Mainx, Felix, 1924. Kultur und Physiologie einiger Euglena-Arten. (Vorl. Mitt.) *Lotos*, **72**: 239.

Mainx, Felix, 1928. Beiträge zur Morphologie und Physiologie der Eugleninen. I. Teil. Morphologische Beobachtungen, Methoden und Erfolge der Reinkultur. II. Teil. Untersuchungen über die Ernährungs- und Reizphysiologie. *Arch. f. Protist.*, **60**: 305.

Pringsheim, E. G., 1912. Kulturversuche mit chlorophyllführenden Mikroorganismen. II. Zur physiologie der Euglena gracilis. *Beitr. z. Biol. d. Pflanzen*, **12**: 1.

Tannreuther, G. W., 1923. Nutrition and Reproduction in Euglena. *Arch. f. Entw. d. Organism.*, **52**: 367.

Ternetz, Charlotte, 1912. Beiträge zur Morphologie und Physiologie der Euglena gracilis, Klebs. *Jahrb. f. wiss. Bot.*, **51**: 435.

Turner, C. L., 1917. A Culture Medium for Euglena with Notes on the Behavior of Euglena. *Anat. Rec.*, **12**: 407.

Zumstein, H., 1900. Zur Morphologie und Physiologie der Euglena gracilis, Klebs. *Jahrb. f. wiss. Bot.*, **34**: 149.

THE MUSCULAR ACTIVITY AND OXYGEN CONSUMPTION OF URECHIS CAUPO

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I. Introduction: Natural History

Urechis caupo, a large marine echiuroid worm recently discovered on the California coast by Fisher and MacGinitie (1928), presents by virtue of its habits of life a unique opportunity for the quantitative study of the interrelations between muscular activity, rate of metabolism and the mechanism of exchange with the environment.

The animal digs and lives in a U-shaped burrow in the mud of shallow estuaries, leaving it only occasionally to construct a new burrow. The upper ends of the burrow open freely to the water. The requisite exchanges with the environment: respiratory, nutritive, excretory and reproductive, are accomplished by the animal forcing a stream of water through the burrow. The movement of water is produced by peristaltic waves in the musculature of the body wall, originating at or near the anterior end, and passing posteriorly. The integument in the region between two consecutive waves is pressed closely against the wall of the tube. Accordingly, water between the integument of the constricted regions and the sides of the burrow is carried posteriorly with the peristaltic wave. The worm from time to time turns around in the tube, thus reversing the direction of the stream.

The mode of feeding is unusual. Near the anterior end of the worm there is a ring of specialized mucous glands. The animal presses the body wall in the region of these glands firmly against the side of the burrow; then, as the glands secrete, it backs away, leaving a tube of mucus attached to the burrow at one end, and to its integument at the other. The peristaltic movements, usually suspended during the formation of the tube, are now resumed, drawing a stream of water through the mucus tube, which acts as a filter. Particles over one micron in diameter are retained. After filtration of water has continued for some time, the worm moves forward, seizes the tube with the proboscis and swallows it whole. Since the food consists of particles included in the detritus of the estuary bottom, this mechanism enables the animal to obtain nutriment without leaving the burrow. The above description of the animal's habits is adapted from Fisher and MacGinitie's account.

Urechis may be kept in the laboratory indefinitely if placed in glass U-tubes of dimensions approximating those of the burrow and if given access to aërated sea water. Several specimens, introduced into such tubes over three years ago by Professor MacGinitie of the Hopkins Marine Station, are now in excellent condition. Their behavior in the laboratory is consistent, as far as is known, with that in their natural habitat.

II. Activity: Volume of Water Pumped Through Tube

The volume of water pumped through the tube in which the animal is living is of interest from two viewpoints: First, since the peristaltic activity of the body wall musculature constitutes by far the greatest part of the muscular activity of the animal, a measure of the volume pumped may be regarded as an approximate indirect measure of the total muscular work. Second, since all exchanges with the environment are mediated through this stream, its measurement yields data relative to the potentially available oxygen and food supplies, and to the facilities for disposal of metabolites and reproductive products.

The method of measurement of the volume pumped is closely related to that devised by Galtsoff (1928) for the study of the flow of water produced by the gills of the oyster.

The apparatus is diagrammed in Fig. 1. One *Urechis* (*A*) was introduced into a glass U-tube (*B*), about 2.5 cm. in diameter, the length of the horizontal segment being 30 cm. and that of each vertical segment 25 centimeters. The tube was placed in an aquarium of approximately 100 liters capacity, through the glass front of which the animal could readily be observed. A stream (*J*) of aërated sea water, filtered free of food materials, entered the aquarium continuously and overflowed from a fixed aperture (*C*), thus maintaining a constant level in the aquarium. The temperature in the aquarium ranged from 15.4 to 18.8° C., the average being 16.9 degrees.

The ends of the U-tube projected above the level of the water in the aquarium. A siphon tube (*D*) admitted water from the aquarium into the artificial burrow at one end. The water, forced through the tube by the work of the worm, passed by means of a second siphon at the other end into an Erlenmeyer suction flask (*E*). The flask was so adjusted that, when filled until water overflowed through the side tube (*F*), the level in the flask was the same as that in the aquarium. Since the level of water in all vessels was the same, the only factor causing water to move was the pumping activity of the worm. The overflowing water was caught in a graduated cylinder (G). Collections were made, as a rule, for five-minute periods. The rate of flow was expressed in cubic centimeters per minute.

Since the animals frequently turn around in the tubes, it was necessary to duplicate inlet siphon, outlet siphon and overflow flask, so that the flow could be measured in either direction. The siphons not necessary at the moment were closed with pinchcocks. For simplicity, there is represented in Fig. 1 only that portion of the apparatus required for measurement of the flow in a single direction.

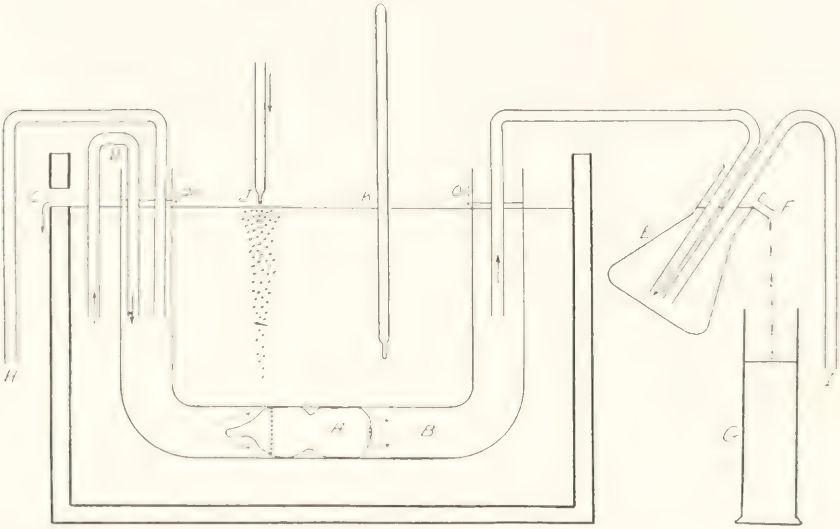

Fig. 1. Apparatus for measurement of volume of water pumped by *Urechis*. Description in text.

The high degree of variability in the rate of pumping which was found made it seem wise to make a number of observations on a few individuals over a considerable period of time rather than a few observations on each of a large number. Accordingly, only two animals were employed, both near the average size of mature worms, *i.e.*, about 60 grams weight. Worm I was perhaps twenty per cent larger than Worm II.[1]

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[1] Professor G. E. MacGinitie of the Hopkins Marine Station, Stanford University, kindly provided me with the following weights of ten mature specimens of *Urechis*:

	Average, grams	Maximum, grams	Minimum, grams
Total body weight	62.5	82.4	35.1
Weight without blood	40.7	53.1	21.1
Weight of blood	21.8	31.3	12.5

The worms were kept in their tubes undisturbed throughout the whole duration of the experiments,—about two months. A period of about a week was permitted for adaptation to their new environment before collection of data was begun.

Results

With the animals under constant standard conditions, the rate of pumping during five-minute periods ranged from 0 to 50 cc. per minute. Two factors in the production of this variability were noted: (1) a consistent increase during the feeding periods—a factor which has been studied in some detail; and (2) long periods of inactivity during which the worm lies in a cylindrical form with integument in contact with the glass over its whole length and shows no movement. Such periods may last from twenty minutes to well over an hour. They are usually terminated by the worm turning around, and then resuming pumping. Concerning the significance of these periods of inactivity no suggestions are offered.

However, even if these sources of variability be excluded by the choice of non-feeding periods during which the worm was continually active, there remains a high unexplained variability. For example, in the case of Worm I in twelve consecutive five-minute periods, in all of which it was active and during which no feeding occurred, the rate of pumping ranged from 8.3 to 29.2 cc. per minute, the mean being 19.3 and the average deviation from the mean, 5.0 cc. per minute.

Average Volumes Pumped

In Table I the results of a number of experiments are tabulated. The first three columns contain the data for the whole period of each observation; the second three columns, the data for the portion of the period during which the worm was feeding; and the third three columns, the data for the portion during which it was not feeding. The last column shows the ratio of the rate of pumping when the worm was feeding to that when it was not feeding. The averages are weighted.

It will be seen that the larger worm averaged throughout the experiments 16.5 cc. per minute; the smaller, 10.2 cc. per minute. The variability among the averages of the experiments is considerable.

TABLE I

Volume of Water Pumped by Urechis under Standard Conditions

Experiment		Total			Feeding			Non-feeding			Ratio $\frac{\text{Feeding}}{\text{Non-feeding}}$
		Vol.	Time	Rate	Vol.	Time	Rate	Vol.	Time	Rate	
		cc.	*min.*	*cc./min.*	*cc.*	*min.*	*cc./min.*	*cc.*	*min.*	*cc./min.*	
Worm I	1	2293	138	16.6	—	—	—	2293	138	16.6	—
	2	2770	149	18.5	524	16	31.7	2246	132	16.9	1.88
	3	4529	438	10.3	—	—	—	4529	438	10.3	—
	4	4435	185	24.0	1856	54	34.4	2579	131	19.7	1.75
	5	3164	129	24.5	1347	37	35.9	1817	91	19.9	1.80
		Average rates		16.5			36.5			14.5	1.81
Worm II	1	2556	150	17.0	1464	58	25.2	1092	92	11.9	2.12
	2	1599	124	12.9	510	22	23.1	1089	102	10.6	2.18
	3	2054	444	4.6	407	32	12.5	1647	412	4.0	3.12
	4	3034	186	16.3	1651	71	23.3	1383	115	12.0	1.94
		Average rates		10.2			22.0			7.2	2.35

Feeding Cycles

The following descriptive data were obtained from a series of twenty-five cycles during which the worms were observed to be feeding.

Frequency of occurrence: Worm I produced a tube on the average 0.7 times per hour; Worm II, 1.5 times per hour.

Duration of feeding periods, from completion of tube to swallowing of tube: averages, Worm I 18.3 minutes; Worm II 8.7 minutes. It is interesting to note that the number of minutes spent in feeding per hour of elapsed time is closely similar in the two animals: Worm I 12.8 minutes per hour; Worm II 13.1 minutes per hour. The larger worm, which fed much less frequently, compensated by greater duration of each feeding period.

Course of activity during the feeding period: Onset: In 18 of the 25 cycles examined there was a decrease in the volume pumped during the five-minute period during which the tube was formed. The actual formation of the tube occupied about 30 seconds—during which time active pumping was suspended. *Course:* In the period during which the tube is present, there was observed in 23 of 25 cycles a clear-cut increase in the rate of pumping as compared with the rate before and after the feeding period. There is often a step-wise increase during the first two or three five-minute periods to an irregular plateau in the curve of pumping rate. *End:* Immediately or within five minutes after the swallowing of the tube there is commonly, but by no means in-

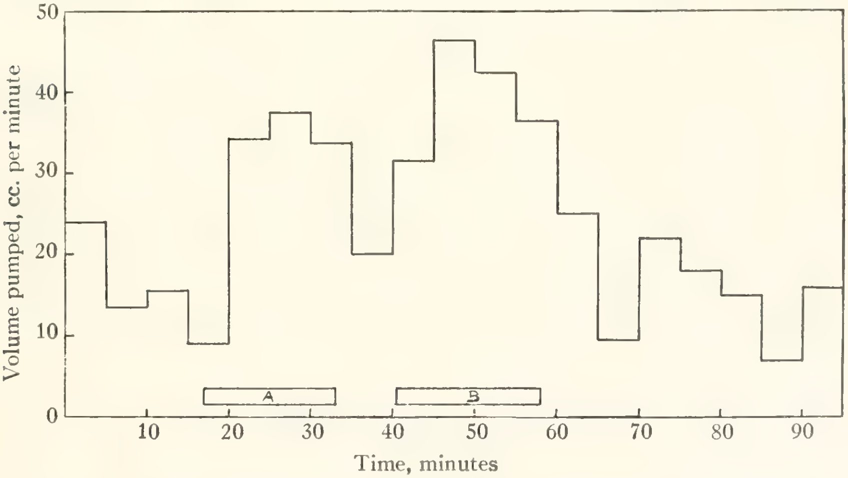

FIG. 2. Rate of pumping by Worm I for a 95-minute period, during which two feeding cycles occurred. The rectangles *A* and *B* indicate the time between the formation and swallowing of the tube in each cycle.

variably, a decrease in the rate of pumping to below 5 cc. per minute. In Fig. 2 there are represented graphically two consecutive cycles which occurred relatively closely together.

Average of activity during the cycle as compared with that during intervals between cycles: These data are included in Table I. The marked increase during feeding is clearly evidenced by the fact that the average ratio of the rate of pumping during feeding periods to that during non-feeding periods is, in Worm I 1.8; in Worm II 2.3. Comparable increases are present in all experiments without exception. In only two of the 25 cycles examined was there no increase. In one of these periods the worm ate the tube within half a minute of making it. In the other, an increase in activity occurred before the feeding was observed. Here it is possible that the tube had been formed earlier and was overlooked for some minutes.

Discussion

Since the current of water pumped through the burrow finds its significance to the organism by making possible exchanges of materials with the external environment, a discussion of its rôle with respect to certain of such materials is pertinent.

(1) *Oxygen.* The respiratory significance of the current has been discussed by Redfield and Florkin (1931), their discussion being based in part upon the results communicated in this paper. These authors point out that the animal utilizes only one-third of the oxygen in the water inhaled into the hind-gut. Accordingly, at the normal rate of oxygen consumption 0.013 cc. per minute, a hind-gut ventilation of 6.9 cc. of water would be necessary. Since the average rate of pumping amounts to about thirteen cubic centimeters per minute, the current is about twice that necessary for the maintenance of normal respiratory relations.

In an attempt to determine the mechanism of adaptation of the worm to waters of low oxygen content, in eight experiments conducted with the apparatus described above, the worms were given access for approximately an hour to sea water boiled until its oxygen content was reduced from about 4.6 to about 2.5 cc. per liter. The oxygen pressure was thus reduced to about seventy millimeters Hg. The pH of the sea water, increased by the boiling, was readjusted to the normal value of 8.2 by addition of a small quantity of dilute hydrochloric acid. The activity of the worms under these conditions was compared with that during similar hour periods, immediately before and after, during which normal sea water entered the tube. No consistent effect was observed, activity being greater in four experiments, unaltered in one and decreased in three. The average, however, is 40 per cent greater than that of the control periods.

As will be described in Part III of this paper, a reduction of oxygen pressure to 70 mm. Hg is accompanied by a reduction of oxygen consumption to about fifty-five per cent of that in normal sea water. The fall in oxygen consumption is of itself adequate to compensate for the decreased amount of oxygen available in the water. This fact, rather than a consistent increase in the current of water pumped through the burrow, appears to be the adaptive response of the animal to water of low oxygen content. For further discussion of this matter, see Redfield and Florkin (1931).

(2) *Food.* The food requirement of the animal (expressed in some such units as calories per hour), together with the food value of the sea water (in calories per liter), determine the volume of water (liters) which would be required to be filtered in order to meet the requirement. It is conceivable that this might be accomplished by means of a continuous stream of constant intensity. However, *Urechis* instead employs the same device as the higher animals, that of periods of intense food-getting activity alternating with periods in which the animal is freed for other activities. Thus, *Urechis* spends only about one-fifth of its time in the obtaining of food. In order to accomplish the required filtration within this restricted time, a relatively high degree of activity is necessary. Unfortunately the data are not available which would make possible an assessment of the significance of the magnitude of the stream for feeding as has been done for oxygen.

Under the conditions of the experiments the worms were provided with sea water so filtered as to be practically devoid of food value. They had been, and were, accordingly, in a state of chronic starvation. Whether this would serve to evoke a maximum intensity of food-getting activities, or would rather, after a time, cause decreased activity and reduced rate of metabolism as occurs in the chronic inanition of mammals (Lusk, 1928), is not known. However, the fact should be borne in mind in any attempt to apply the data to *Urechis* in its normal habitat.

The stimulus provoking the feeding reaction is not known. That it is not of external origin is shown by the fact that the two worms, in similar tubes side by side in the aquarium, subjected to the same environmental influences, including light, jarring, etc., and receiving the same sea water, carried out their feeding reactions totally independently of each other in time. The stimulus is probably of internal origin.

If, during the period of feeding, a relatively minor mechanical disturbance be brought about, such as gently moving the inlet siphon tube, the worm abruptly stops pumping, casts loose the mucus tube and backs down into the horizontal part of the U-tube.

III. OXYGEN CONSUMPTION

The oxygen consumption of *Urechis* was determined by two methods which yielded similar results: (1) a worm, active in a U-tube under the conditions described above, pumped water from the aquarium into the Erlenmeyer suction flask which had previously been filled with mineral oil, so displacing the oil with water. The oxygen content of the water in the U-tube in front of the worm (incoming water) and of that in the flask at the end of the period (outgoing water) was determined by the method of Winkler (1888), samples being withdrawn by means of siphons (*H* and *I* of Fig. 1) without disturbing the animal in any way. Knowing the oxygen content of the incoming and outgoing waters and the volume pumped, the oxygen consumption could be readily calculated.

(2) A worm was placed in a jar containing approximately three liters of sea water, over the surface of which a layer of mineral oil about one-fourth inch thick was floated. Samples of water were withdrawn at intervals by means of a siphon and their oxygen content determined by the Winkler method.

Results: First Method

In Table II are tabulated the oxygen consumption (in cc. per minute), the oxygen partial pressure in the incoming water (mm. Hg), the oxygen content of the incoming and of the outgoing waters (cc. per liter), and activity or volume of water pumped (cc. per minute). The experiments are arranged in order of increasing activity. It will be noted that there is a general tendency for the oxygen consumption to increase with increasing activity, as would be expected. There is, in these experiments, no consistent relation between the oxygen partial pressure of the incoming water and the oxygen consumption of the animals. The oxygen consumption rate of the two worms is almost identical, being 0.0130 and 0.0136 cc. per minute respectively.

Second Method

The oxygen consumption of the animals kept in jars under oil was, during the initial period of each experiment, as follows: 0.0141, 0.0173, 0.0281 and 0.0120; average, 0.0179 cc. per minute. These values are definitely higher than those obtained for the oxygen consumption of worms in the U-tubes. The difference is attributable to the exaggerated peristaltic activity exhibited by the animals in the jars. Apparently, the absence of the normal contact of the integument serves to activate the animal's movements. These values are accordingly considered less representative of the metabolic rate under normal conditions than those obtained by the U-tube method.

A comparison of the oxygen consumption of *Urechis* with that of closely related forms determined by other workers follows.

Table II

Oxygen Consumption of Urechis in U-tubes

Animal	Oxygen Consumption	Incoming water		Outgoing water	Activity
		Oxygen Partial Pressure	Oxygen Content	Oxygen Content	
	cc./min.	*mm./Hg*	*cc./liter*	*cc./liter*	*cc./min.*
I.	0.0091	138.2	4.90	2.80	4.3
	0.0085	127.2	4.51	3.51	8.5
	0.0154	130.0	4.61	3.17	10.7
	0.0123	191.8	6.80	6.10	19.5
	0.0150	93.3	3.31	3.01	50.0
	0.0178	94.2	3.34	3.02	54.3
Average.......	0.0130				
II.	0.0097	133.7	4.74	4.18	17.9
	0.0118	96.0	3.40	2.92	24.7
	0.0194	96.2	3.41	2.92	45.2
Average.......	0.0136				

Comparison of Metabolic Rates of Certain Invertebrates

Animal	Author	Oxygen Consumption *cc. O_2 gm./min.*
Lumbricus	Averaged results of Thunberg, Lesser and Konopacki, quoted by Krogh (1916).....	0.00189
Glycera siphonostoma	Cohnheim (1911–12)......................	0.00123
	Montuori (1913)..........................	0.00025
Hirudo	Rogers (1927)............................	0.00052
Sipunculus nudus	Cohnheim (1911–12)......................	0.00082
Urechis	Present author...........................	0.00021

Urechis thus possesses a metabolic rate of magnitude comparable to related forms but distinctly lower. This is in part attributable to the fact that this animal has a blood volume disproportionately great for its size as compared with allied forms. Thus, an average-sized animal weighing 62.5 grams possesses blood weighing 21.8 grams. Although the corpuscles are true cells, it is doubtful whether their metabolism would give to the blood a rate of oxygen consumption per gram comparable to that of the fixed tissues. If the total oxygen consumption be calculated on a basis of fixed tissue weight, it becomes 0.00033 cc. per gram per minute.

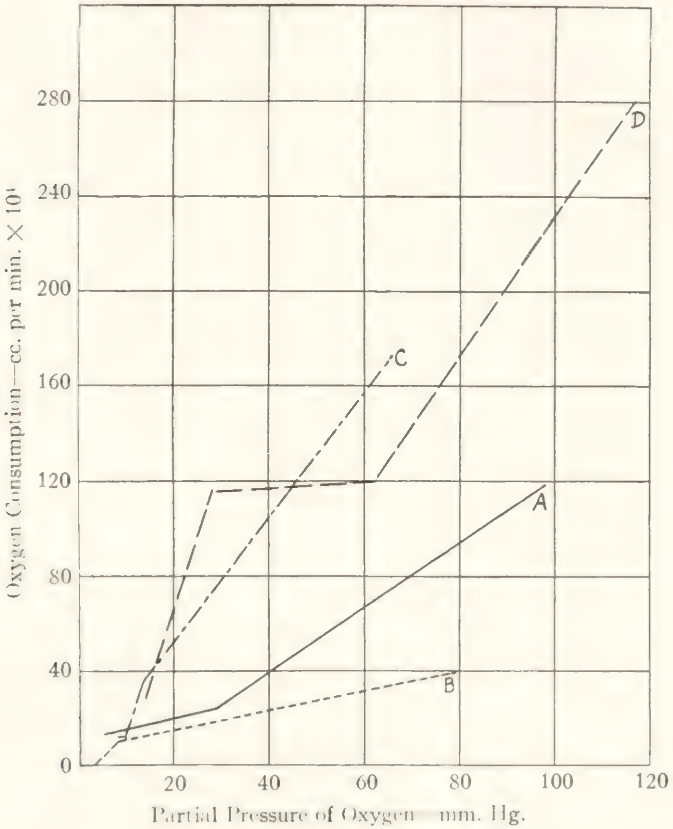

FIG. 3. Relation of oxygen consumption to oxygen partial pressure. *Urechis*. The letters at the extremities of the curves indicate the correspondence of the curves to the experiments reported in Table III.

Oxygen Consumption and Oxygen Pressure

In the experiments in which worms were placed in jars containing approximately three liters of sea water, the oxygen content of the water was determined at intervals as it fell due to the metabolism of the animals. In several cases the rate of fall was accelerated by placing three worms in a jar instead of a single one. When the oxygen content had fallen to about 0.2 cc. per liter, the experiment was discontinued.

The worms were still active, as shown by persistence of spontaneous peristaltic activity.

To determine the oxygen partial pressure of the sea water, the oxygen content was plotted against time, and the oxygen content read off the curve for the middle of each period between successive samplings. Under the conditions of the experiments, namely, at a temperature of 17° C., an atmospheric pressure of 760 mm. Hg, and a sea water chloride content of 19 grams per liter, the oxygen content of water in equilibrium with atmospheric air is 5.66 cc. per liter (measured at N. T. P.). (Fox, 1907.) Under these conditions the oxygen partial pressure is 159.6 mm. Hg. The partial pressure of any sample of such water of which the oxygen content is known can be readily calculated by application of Henry's law.

Table III

Oxygen Consumption and Oxygen Pressure

Experiment and period number	Duration of period		Initial O_2 content	O_2 pressure at mid-period	Oxygen consumption
	hrs.	*min.*	*cc./l.*	*mm.Hg*	*cc./min./worm*
A: 1	21	30	5.15	97.6	0.0118
2	44	00	1.78	29.0	0.0024
3	6	30	0.28	5.6	0.0013
4	—	—	0.12	——	——
B: 1	24	00	5.19	79.0	0.0039
2	4	10	0.42	8.5	0.0011
3	22	00	0.18	3.9	0.0001
4	—	—	0.10	——	——
C*: 1	2	15	3.60	66.0	0.0173
2	1	00	1.08	24.5	0.0063
3	1	05	0.66	14.4	0.0037
4	1	00	0.37	9.9	0.0006
5	1	05	0.33	8.5	0.0006
6	—	—	0.28	——	——
D: 1	1	15	5.24	116.8	0.0281
2	2	05	3.04	62.6	0.0120
3	1	00	1.40	28.2	0.0115
4	0	55	0.61	14.4	0.0028
5	—	—	0.42	——	——

* At the conclusion of Experiment B, the greater part of the water was removed from the jar and fresh water substituted. The earlier parts of Experiment C then represent post-anoxybiotic metabolism, the more active oxygen consumption suggesting that an "oxygen debt" was being made up.

In Experiment A one worm was used, in the remaining experiments, three.

To determine the rate of oxygen consumption, the change of oxygen content in any period was multiplied by the volume of water then present, due allowance being made for the volumes removed in sampling.

These data are presented in Table III and are represented graphically in Fig. 3. It is clear that, under the conditions of these experiments, the oxygen consumption of the worms decreased in an approximately linear manner with the oxygen pressure throughout the range of 116.8 to 3.9 mm. Hg. It has already been noted that the oxygen consumption of worms active in U-tubes bore no consistent relation to oxygen pressure at least over the range of 138.2 to 93.3 mm. Hg. These results are not necessarily in conflict, for (1) the ranges of pressure are not the same, overlapping by some 25 mm. Hg, and (2) the experimental conditions differed significantly.

Discussion

The large literature which has accumulated relative to the influence of oxygen pressure on oxygen consumption has been most recently reviewed by Helff and Stubblefield (1931), who list the animals studied and classify their responses, and by Buchanan (1931), who gives a short historical survey of theoretical interpretations of the relationship.

Various reasons have been given for the fall of oxygen consumption which accompanies a decrease in oxygen pressure below a critical value. The applicability of these suggestions to the respiratory mechanism of *Urechis* will be discussed:

(1) *Oxygen deficiency in metabolizing cells due to inadequate transport of oxygen to them.* Accepting the doctrine of Pflüger (1872) that the oxygen consumption rate of cells is determined by their own organization and is independent of the concentration of oxygen in their immediate milieu, provided that the latter be above zero, Krogh (1916) concluded that the decline in oxygen consumption with decreasing oxygen pressure of the external medium was due to the attainment by successive groups of cells of an oxygen-free state, with consequent cessation of metabolism. This condition might result either from the absence of adequate respiratory and circulatory mechanisms, or from relatively slow diffusion of oxygen into the region of active oxidation. This conclusion has been questioned by, among others, Gerard (1931), who has shown by careful mathematical analysis of the interrelations between the oxygen consumption rate, pressure and diffusion rate in the case of unicellular organisms that Pflüger's assumption is incompatible with experimental results. He concludes that oxygen consumption must change with oxygen pressure over a significant range in that

region where the oxidation is actually taking place. Factors such as alteration of permeability to oxygen or change in concentration of oxidative enzymes (Buchanan, 1931), or decreased adsorption of oxygen on catalysts of biological oxidation (Shoup, 1929) have been suggested as possible mechanisms.

In *Urechis,* the respiratory and circulatory mechanisms, which have been quantitatively analyzed by Redfield and Florkin (1931), are quite effective, and possess large "factors of safety." The current of water through the burrow carries about six times as much oxygen as is used in metabolism. Only one-third of the oxygen taken into the hind-gut is utilized. The attainment of equilibrium between hind-gut water and blood, and between blood and active tissues, is facilitated by the peristaltic movement of both hind-gut and body-wall. The maximum distance from blood to muscle rarely exceeds one millimeter. The rate of metabolism is such that only one-sixtieth of the blood oxygen content is used per minute. The hemoglobin, fully saturated at the normal physiological oxygen pressure, becomes an oxygen transporter at lower oxygen pressures. From these considerations it seems justifiable to conclude that deficient oxygen transport to the active cells is not responsible for the fall in oxygen consumption with falling oxygen pressure in the sea water provided to the animal.

(2) *Accumulation of carbon dioxide* might decrease oxygen consumption, either of itself or by increasing the hydrogen ion concentration. In Experiment D, the three worms decreased the oxygen content of the 2664 cc. of sea water by 11.0 cc. at the end of 4.35 hours, by which time the oxygen pressure was 14.4 mm. and the oxygen consumption reduced to 10 per cent of its initial value. Assuming an R. Q. of 1, 11.0 cc. of carbon dioxide would be formed, which, in the volume of 2664 cc., would increase the carbon dioxide concentration by 0.00017 M. From the data of McClendon (1917), it may be estimated that this, in normal sea water, would cause an alteration of 0.05 mm. Hg in carbon dioxide pressure, and a pH decrease of 0.07.

Moderate increases in CO_2 pressure in the case of sea urchin eggs (Warburg, 1910), the lobster *Homarus americanus* and the sand worm *Nereis virens* (Amberson, Mayerson and Scott, 1924), and certain aquatic insects (Hiestand, 1931) did not decrease oxygen consumption. Root (1930) found in fertilized *Arbacia* eggs that each 10 mm. Hg of CO_2 pressure reduced oxygen consumption by 21 per cent. It is obvious that the change produced by 0.05 mm. CO_2 pressure would be negligible. Although Burfield (1928), using plaice eggs, and Fowler (1931), using *Daphnia,* found that CO_2 depresses oxygen consumption, their experiments are not described in a manner permitting evaluation of the small change under consideration here.

It seems improbable that accumulation of carbon dioxide was a major factor in the depression of oxygen consumption observed in *Urechis*.[2]

(3) *Alteration in the intensity of muscular activity.* No careful studies of the relation of the degree of spontaneous muscular activity to the oxygen pressure of the external environment have been found in the literature, although the necessity of controlling this factor is well recognized. Attempts to remove its influence by anesthesia (Gaarder, 1918; F. G. Hall, 1929) introduce new complexities, as the anesthetic used, ethyl urethane, is known to depress basic metabolism (Field and Field, 1931).

As has already been stated, reduction to 70 mm. Hg of the oxygen pressure of the water supplied to *Urechis* in U-tubes produced consistent changes in neither the degree of muscular activity nor the oxygen consumption. On the contrary, the oxygen consumption did tend to vary with the muscular activity. In this case, any influence which lowered oxygen pressure may have had upon oxygen consumption was overshadowed by the influence of muscular work. It is possible that, had lower oxygen pressures been employed, an influence of this factor might have been uncovered.

Unfortunately, no quantitative observations of the muscular activity of the worms in jars were possible.

(4) *Alteration in character of metabolism.* It is possible that the exothermic processes yielding energy for basic and functional metabolism, such as the decomposition of glycogen with the formation of lactic acid, might proceed throughout the period at a relatively constant rate, while the reconstitutive processes, which are directly or indirectly dependent on oxidations involving molecular oxygen, might lag behind, with the consequent accumulation of an "oxygen debt." In a single experiment Table III, (Experiment C) some evidence of the occurrence of such a process was obtained.

From the facts considered above it does not seem legitimate to draw any positive conclusions as to the reason for the depression of oxygen consumption accompanying the decreased oxygen content of the water. The rôle of muscular activity and of the qualitative aspect of metabolism merit further investigation.

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[2] Since Redfield and Florkin (1931) have shown that the oxygen dissociation curve of *Urechis* hemoglobin is not influenced by the carbon dioxide pressure, criticism, such as Keys (1930) has urged against the work of F. G. Hall (1929) and others on the grounds that carbon dioxide would interfere with oxygen transport, is inapplicable to the present investigation.

SUMMARY

The greater part of the muscular activity of the echiuroid worm *Urechis caupo* is involved in pumping a current of water through its U-shaped burrow. The magnitude of this current was studied in artificial burrows, food-free water being supplied to the animals. When the animal is not feeding, the current amounts to about eleven cubic centimeters per minute. During feeding periods, the rate of pumping rises to about twenty-nine cubic centimeters per minute. The frequency, duration and course of activity during these feeding periods has been studied. The significance of the stream in relation to provision of oxygen and food is discussed.

The oxygen consumption of the animals in U-tubes amounts to 0.00021 cc. per gram per minute, being comparable to that of related forms. It is independent of the oxygen pressure down to a value of 70 mm. Hg.

The oxygen consumption of the worms when placed in covered jars decreases with falling oxygen pressure throughout the range investigated, 115 to 4 mm. Hg. The reasons for this fall are discussed.

The author wishes to acknowledge his indebtedness to Mr. G. E. MacGinitie for providing the animals used and for much useful advice in their handling, to Dr. A. C. Redfield for suggestions which made possible correlation of this work with that being carried out simultaneously by himself and Florkin on the same animal, and to Fr. L. Rudolph, Mr. C. Watson and Mr. A. Fryer for assistance in carrying out the experiments.

BIBLIOGRAPHY

AMBERSON, W. R., MAYERSON, H. S., AND SCOTT, W. J., 1924. *Jour. Gen. Physiol.*, **7**: 171.

BUCHANAN, J. W., 1931. *Biol. Bull.*, **60**: 309.

BURFIELD, S. T., 1928. *Brit. Jour. Exper. Biol.*, **5**: 177.

COHNHEIM, O., 1912. *Zeitschr. physiol. Chem.*, **76**: 298.

FIELD, J., 2D, AND FIELD, S. M., 1931. *Proc. Soc. Exper. Biol. and Med.*, **28**: 995.

FISHER, W. K., AND MACGINITIE, G. E., 1928. *Ann. Mag. Nat. Hist.*, Ser. 10, **1**: 199 and 204.

FOWLER, J. R., 1931. *Physiol. Zool.*, **4**: 214.

FOX, C. J. J., 1907. *Pub. de Circonstance.* Copenhagen. No. 41.

GAARDER, T., 1918. *Biochem. Zeitschr.*, **89**: 94.

GALTSOFF, P. S., 1928. Bull. Bur. Fish., **44**: Document No. 1035.

GERARD, R. W., 1931. *Biol. Bull.*, **60**: 245.

HALL, F. G., 1929. *Am. Jour. Physiol.*, **88**: 212.

HELFF, O. M., AND STUBBLEFIELD, K. I., 1931. *Physiol. Zool.*, **4**: 271.

HIESTAND, W. A., 1931. *Physiol. Zool.* **4**: 246.

KEYS, A. B., 1930. *Bull. Scripps, Inst.*, Tech. Ser., **2**: 307.

KROGH, A., 1916. The Respiratory Exchange of Animals and Man. Longmans, Green and Co. London.

LUSK, G., 1928. The Science of Nutrition, 4th Ed. Ch. IV. Saunders, Philadelphia.

McClendon, J. F., 1917. *Jour. Biol. Chem.*, **30**: 265.
Montuori, A., 1913. *Arch. ital. Biol.*, **59**: 213.
Pflüger, E., 1872. *Pflüger's Arch.*, **6**: 43.
Redfield, A. C., and Florkin, M., 1931. *Biol. Bull.*, **61**: 185.
Rogers, C. G., 1927. Textbook of Comparative Physiology. McGraw-Hill Book Co. New York.
Root, W. S., 1930. *Biol. Bull.*, **59**: 48.
Shoup, C. S., 1929. *Jour. Gen. Physiol.*, **13**: 27.
Warburg, O., 1910. *Zeitschr. physiol. Chem.*, **66**: 305.
Winkler, L. W., 1888. *Berichte Chem. Ges.*, **21**: 2843.

THE BLOOD PIGMENTS OF URECHIS CAUPO

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The echiurid *Urechis caupo* was discovered by Fisher and MacGinitie and is an abundant inhabitant of the Monterey Bay in California.[1] One of its interesting features is its richness in hemoglobin. This has been the subject of an extended study by Redfield and Florkin.[2] It is a peculiarity of this invertebrate that its hemoglobin is contained within the blood cells and none in the blood fluid. Another localization of the hemoglobin is the muscles, which are not vascularized but contain all hemoglobin within the muscle cells; and, furthermore, the dorsal nerve chord appears red with hemoglobin. There are, however, several other particular aspects with respect to the blood pigment which are to be presented in this paper. In part, they are concerned with changes, probably according to the age or to the seasons, which could not be fully studied during one season. The description of these changes will be presented as they appeared to be and may be subject to modifications as further studies may be extended over a longer period of time.

The animals at our disposal varied in length, in the contracted state, from 3 to 10 inches. Accordingly, the blood content of the body cavity varied from 10 to 30 cubic centimeters. The color of the blood varies, from the purest oxyhemoglobin-red to the darkest brown-black or a black like chinese ink, even after complete saturation with oxygen. This variation of the blood is a very striking feature and obviously has a definite physiological significance.

Red blood was encountered in some few of the smallest individuals, and in some of the very largest sex-mature females. The majority of the individuals, of medium size, contained brown or brown-black blood. The blackest blood ever encountered was that of a very large sex-mature male. The cause of the difference in color is revealed by a microscopic examination. The red color of the blood is due to hemoglobin homogeneously distributed within the blood cells. Whenever the color is brown, besides this hemoglobin there is another, granular, pigment of brown color within the cells which will be proved to be hematin. The description of the changes in these pigments may be presented according to ideas developed during a study of two months. This may not be sufficient to make sure of all details, and the whole picture may be liable to some modifications upon more extended studies.

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[1] Fisher, W. K., and MacGinitie, G. E., (1928), *Ann. and Mag. Nat. Hist.*, Ser. 10, vol. **1**, p. 199 and p. 204.

[2] Redfield, E., and Florkin, M., 1931. *Biol. Bull.*, **61**: 185.

We start from a pure red blood in a young animal, recalling the fact that not every small animal of our material contained the blood in the red condition. In such an animal, the blood cells are spherical, about 10–15 μ in diameter. The protoplasm is diffusely yellowish-green with hemoglobin and, besides, rather tightly packed with colorless granula of regular spherical shape, of a rather high refractory index,—though not so high as that of fat drops,—and about 1 μ in size. No nucleus is visible in the fresh preparation but a nucleus becomes visible after fixing and staining (fixed in acetone and stained with safranin). The nucleus is small, in the centre of the cell, and contains a distinct nucleolus. Besides these cells, there is another kind, usually somewhat smaller, much less numerous, containing yolk-yellow droplets of a considerable size which often are conglomerated into a mulberry-like packet.

When the blood becomes brown, the granula of the hemoglobin-containing cells are no longer colorless but are stained with a brown pigment The granula, then, are no longer quite uniform in size and spherical in shape, but somewhat more irregular. The size of the cell is the same as in the red blood of young worms. This aspect was most common among our material.

Now we come to the large sex-mature worms. Here a difference arises according to the sex. One feature is common for both sexes. The corpuscles become larger, up to 35 μ in diameter, and more variable in size. In the males, the brown pigment no longer stays exclusively within the granula, but is more homogeneously scattered over the cell so that the hemoglobin color is overshadowed and can be detected only by the spectroscope. The granula at the same time undergo a disintegration. They swell and have indistinct contours, being, as it were, dispersed into a turbid mass without definite structure. At the same time very small, spherical, quite black pigment granula, very dense in structure, and not very numerous, are formed within the cell. We do not know whether the development will go beyond this stage, but it appears as though all hematin would gradually disappear and in part be converted into the dense black pigment.

The disintegration of the brown granula takes place in the females also; but it does not lead to the formation of black granula within the blood cells. Rather is the blood cell gradually deprived of any pigment except for the hemoglobin. Instead, a pigment is formed within the eggs, and there can be little doubt that the brown blood pigment is the source of the black egg pigment.

The egg is a very large cell of almost the same aspect as that of *Asterias*, also with respect to the size and shape of the nucleus. After insemination, the nucleus disappears and the polar bodies are formed. In the protoplasma of the egg a very fine dust of pigment granula is scattered. The number of these granula is not very large so that the eggs show macroscopically only a very slight yellowish-grey shade. These pigment granula are in part black, in part somewhat more dark red. The black pigment has the same shade as the one in the erythrocytes of the male, the difference being only that the black granula in the eggs are usually smaller than those in the male erythrocytes. Upon

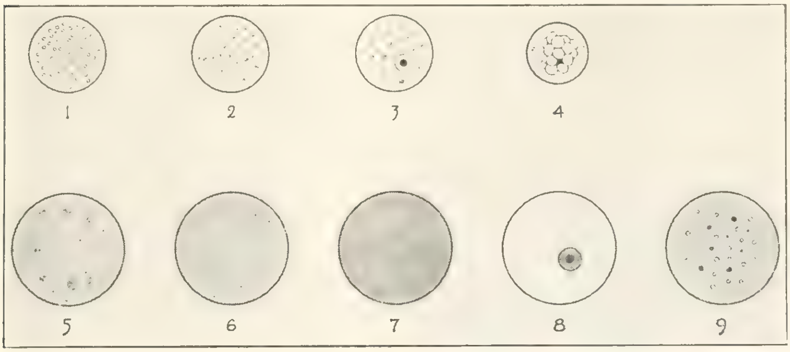

FIG. 1. Blood cell with regular, colorless granula of a relatively high refractory power. Hemoglobin is diffusely dissolved in the protoplasma, not in the granula. Fresh preparation.

FIG. 2. Blood cell with hematin-stained granule. Granula brown, protoplasma yellowish-green with hemoglobin. Fresh preparation.

FIG. 3. The same, dry smear, fixed with acetone and stained with safranin. Nucleus with nucleolus.

FIG. 4. Smaller cell with yolk-yellow droplets, containing sometimes also a few hematin granula. Fresh preparation.

FIG. 5. Larger blood cell of a sex-mature female, with only a few hematin granula. The whole protoplasma is diffusely yellowish-green with hemoglobin, with no distinct structure. Fresh preparation.

FIG. 6. Larger blood of a sex-mature female, yellowish-green with hemoglobin, without any hematin, very fine colorless granula of low refractory power. Fresh preparation.

FIG. 7. The same, without any distinct granular structure, pure hemoglobin shade in the whole protoplasma. Fresh preparation.

FIG. 8. The same, dry smear, fixed with acetone and stained with safranin, showing the nucleus.

FIG. 9. Blood cell of an old male, black pigment besides colorless granula. The protoplasma is diffusely light brown. Fresh preparation.

confronting the fact that the black pigment is met, in the males, only within the blood cells and never in the sperm, and in the females only in the eggs and never in the blood cells, the interpretation seems unavoidable that the brown pigment is the mother material for the black one and is utilized for the eggs in the female, but remains in the blood cells of males.

It is likely that the brown pigment (which will be identified with hematin) is converted, in part, into the black granular pigment, and also in part into hemoglobin again. This latter conclusion is suggestive because the sex-mature females with purely red blood have blood cells of a much larger size than younger animals and yet these cells certainly do not contain the hemoglobin in a lower concentration.

The blood cells can be hemolyzed by a copious amount of distilled water, or in the undiluted blood, by some drops of ether, or better, by gently shaking with a drop of octyl alcohol. The granula described above will float isolated in a preparation of the laked blood. The colorless granula remain as individuals, very often also the brown granula, though these may also be disintegrated to finer pigment granula of yellow brown color. All transitions can thus be observed from colorless granula to partially and completely stained granula.

The chemical behavior of the hemoglobin has been fully described by Redfield and Florkin. It agrees in all its reactions and in all optic properties with mammalian hemoglobin. It can be separated from the brown pigment simply by centrifuging the blood hemolyzed with a drop of octyl alcohol. The brown pigment is entirely insoluble and forms the main part of the cake-like sediment, whereas the hemoglobin is dissolved in the supernatant liquid. The brown pigment can be extracted from the cake-like sediment in the following way: The cake is first extracted with acetone (or ether). A yolk-yellow pigment is herewith extracted which is present either in the blood fluid or in the yolk-yellow cells described above. When this extraction is complete, another extraction is performed with acetone (or ether) containing acid (glacial acetic acid or some drops of strong HCl). Hereupon the brown pigment goes into solution and reveals the characteristic bands of acid hematin. When this solution is reduced, either by shaking with solid sodium hydrosulfite, or with platinum asbestos and hydrogen, and pyridine is added, the characteristic spectrum of pyridine-hemochromogen arises with its very distinct two bands even in highest dilution. The brown pigment has herewith identified itself with hematin. The pyridine-hemochromogen prepared from the hemoglobin, by treatment with acid aceton, reduction and addition of pyridine, is spectroscopically identical with the one prepared in the same way from the hematin granula. Both from the hemoglobin and from the brown granular pigment Teichmann's crystals could be obtained.

It may be alluring to venture an interpretation of the physiological significance of the changes occurring in the blood of this animal. We prefer, however, to refrain from such an interpretation until experiments of a more physiological nature are available.

ON THE RESPIRATORY FUNCTION OF THE BLOOD OF THE SEA LION

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The capture of a Steller's sea lion, *Eumetopias stelleri,* at the Hopkins Marine Station has afforded an opportunity to obtain certain data on the conditions of equilibrium between the blood of an aquatic mammal and the respiratory gases, which have not been available before. The animal, which proved to be an old female, was blind, and having been wounded with a rifle shot while it sat on the rocks in front of the station, was secured with a gaff as it attempted to escape and brought to shore. There it was killed by severing the great vessels in the neck and a sample of 200 cc. of blood was collected as it flowed from the wound. The animal was somewhat emaciated, but was not in a starving condition as evidenced by a quantity of fish in its stomach and the abundance of fat in the lacteals. The bullet wounds were found to be limited to bony and muscular structures and had not caused extensive bleeding. We are indebted to Dr. G. E. MacGinitie for placing the blood at our disposal.

The blood was prevented from clotting by the addition of potassium oxalate; and was kept on ice during the subsequent sixteen hours in which measurements were made. Samples were equilibrated with various gas mixtures in a water bath at 38° C. for 20 minutes and then analyzed for oxygen or carbon dioxide with the Van Slyke " constant volume " apparatus. The gas mixtures were subsequently analyzed with the Haldane gas analysis apparatus. The resulting data are recorded in Tables I and II. In order to correct the observed oxygen contents for the dissolved oxygen, an absorption coefficient of $\alpha = 0.022$ was assumed. The volume of erythrocytes in the blood was determined with the centrifuge and proved to be 29 per cent of the total volume of the blood.

Since all the observations recorded above were made in a short period of time upon a single sample of blood, there was no opportunity to check the results, which must in consequence be regarded as provisional.

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[1] Fellow of the C. R. B. Educational Foundation.

TABLE I

Data on the equilibrium of sea lion's blood with oxygen. Temperature 38° C.

Carbon dioxide pressure	Oxygen pressure	Oxygen content	Oxygen dissolved	Oxygen as oxyhemoglobin	Saturation
mm. Hg	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*
42.20	27.53	5.23	0.08	5.15	25.9
42.80	32.00	6.75	0.09	6.66	33.5
47.00	43.00	10.95	0.12	10.83	54.5
26.20	14.32	2.12	0.04	2.08	10.5
21.20	34.55	10.70	0.10	10.60	53.4
24.20	27.55	12.75	0.08	12.67	64.8
24.30	33.30	11.56	0.10	11.46	57.7
106.50	61.55	11.67	0.18	11.49	58.0
air	air	20.40	0.45	19.95	100.5
air	air	20.21	0.45	19.76	99.5

TABLE II

Data on the equilibrium of sea lion's blood with carbon dioxide. Temperature 38° C.

	Oxygen pressure	Carbon dioxide pressure	Carbon dioxide content
	mm. Hg	*vol. per cent*	*vol. per cent*
Oxygenated	150 ca.	45.60	39.15
	150 ca.	14.45	22.45
	150 ca.	46.40	38.01
Reduced	8.20	54.90	46.00
	4.20	45.20	43.20

DISCUSSION OF RESULTS

In the blood of an aquatic mammal it is reasonable to look for conditions which favor the circulation of oxygen to the muscles in order to maintain the great energy expenditure required for rapid progression through a viscous medium. One may also anticipate an increased oxygen capacity to enable the animal to remain longer under water. In the present instance the oxygen content of the blood when equilibrated with air was 19.8 volumes per 100 volumes of blood. This was not a greater oxygen capacity than commonly occurs in man and other mammals. The volume occupied by the erythrocytes was only 29 per cent of the total blood, a figure much less than that commonly found in active terrestrial mammals. Each cubic centimeter of corpuscles combined with 0.68 cc. oxygen. Drastich (1928) has found that in a large number of domestic mammals the concentration of hemoglobin in the erythrocytes is approximately the same, being about 32 grams hemoglobin per 100 cc. blood corpuscles. Taking one gram of hemoglobin to combine with 1.34 cc. oxygen, each volume of corpuscles combines with 0.43 cc. oxygen. It appears then that the sea lion corpuscles combine with about one and one-half times as much oxygen as do those of the domestic mammals, *i.e.*, the hemoglobin is just that much more concentrated within them. We suspect that the blood under examination may represent a somewhat anaemic condition and that the blood of a younger and more vigorous sea lion would exhibit a higher cell volume and oxygen capacity. Whether or not that is the case, there can be little doubt that the unusual concentration of hemoglobin in the corpuscles of this specimen represents an advantageous condition in that it minimizes the work which must be done by the heart in circulating oxygen through the muscles.

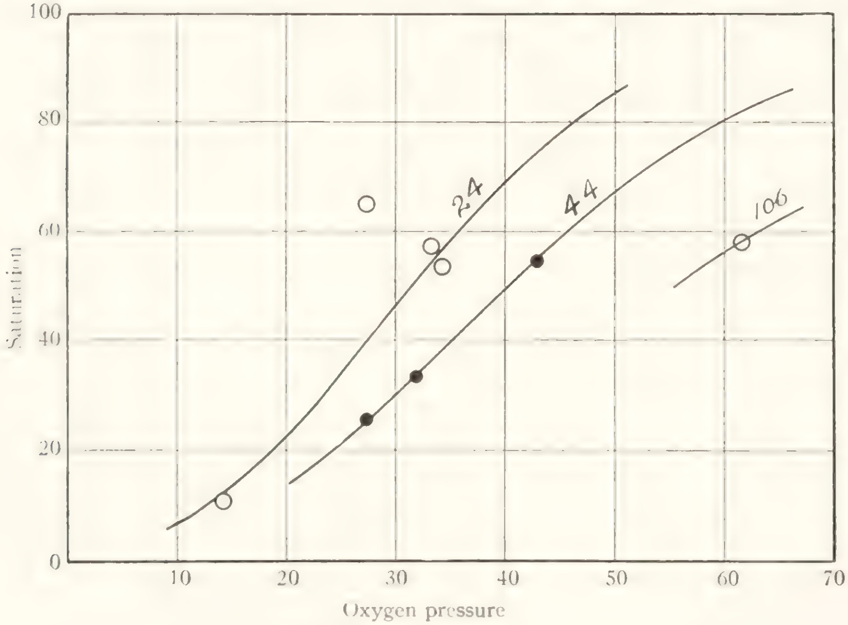

FIG. 1. Oxygen dissociation curves of blood of sea lion at 38° C. The approximate pressures of CO_2, in mm. Hg, at which the blood was equilibrated are indicated by the numbers above the curves. Ordinate, percentage of saturation with oxygen; abscissa, oxygen pressure in mm. Hg.

Sudzuki (1924) reports in the case of porpoise blood (Tümmlerblut) oxygen capacities of 42.5 and 45.1 volumes per cent. The erythrocyte count in the animals studied varied between 8.4 and 11.2 million per cubic millimeter. Since the erythrocytes of the Cetacea are slightly larger than those of man (Marimoto, Takata, and Sudzuki, 1921), it would appear that in the porpoise the increased oxygen-carrying power is accomplished by increasing the number of blood corpuscles rather than by augmenting the concentration of hemoglobin within the corpuscles.

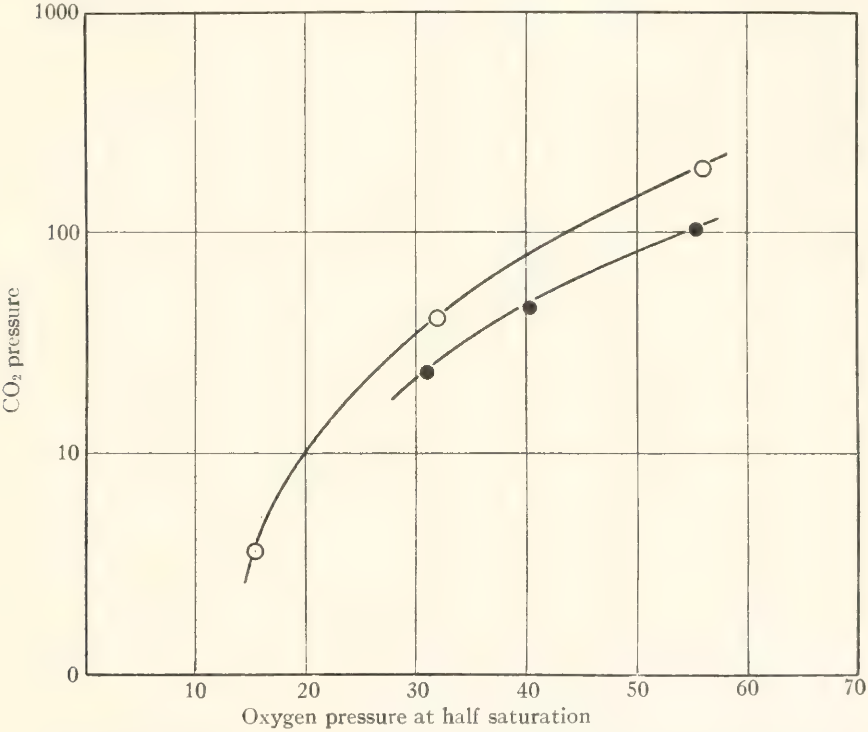

Fig. 2. Oxygen pressures at which the blood of the dog, upper curve, and of the sea lion, lower curve, are half saturated with oxygen in the presence of varying quantities of CO_2. Ordinate, pressures of CO_2 in mm. Hg plotted on a logarithmic scale; abscissa, oxygen pressure in mm. Hg at half saturation.

The Oxygen Dissociation Curve

In Fig. 1 curves are presented which indicate the general nature of the equilibrium of sea lion blood with oxygen at various carbon dioxide pressures. The general form and distribution of the curves resembles that of the blood of other mammals. In order to compare equilibrium conditions in the case of the sea lion with those characterizing the blood of the dog, the pressures of oxygen at which the hemoglobin is half saturated have been plotted in Fig. 2 against the corresponding carbon dioxide pressures. Similarly, a curve has been drawn representing this relation in the case of the dog's blood from data kindly supplied by Dr. D. B. Dill. It appears that oxygen is held at somewhat higher tensions in the blood of the sea lion than in that of the dog. The difference between the two species is not greater than that exhibited by various specimens of human blood, however. The advantage of this difference, in so far as it exists, in facilitating the rapid diffusion of oxygen into the active muscle fibers, is obvious. The slope of the curves also indicates that a given change in CO_2 tension will cause a greater change in oxygen tension in the case of the sea lion,—again a condition favoring the respiratory exchange.

The Carbon Dioxide Equilibrium

The data in Table II serve to demonstrate the essential facts regarding the equilibrium of carbon dioxide with the blood. If the data are plotted, it will be found that the usual type of CO_2 dissociation curve can be drawn through the points. The carbon dioxide combined at any pressure is somewhat less than in the case of dogs studied in Dr. Dill's laboratory. This condition may very probably be due to the presence of lactic acid in the blood resulting from the struggles of the sea lion in the course of its capture.

The difference in CO_2 content of oxygenated and reduced blood is similar to that of the blood of other mammals.

Summary

The blood of a sea lion, *Eumetopias stelleri,* was found to have an oxygen capacity of 19.8 volumes per cent.

The erythrocytes composed 29 per cent of its volume.

One volume of erythrocytes combined with 0.68 cc. oxygen, indicating a hemoglobin concentration 50 per cent greater than that found in domestic mammals.

The oxygen dissociation curves constructed at various pressures conform to the usual mammalian type, but indicate that oxygen may be held at slightly higher pressures than in the case of dog blood.

The carbon dioxide equilibrium is in no way remarkable and exhibits the usual difference between oxygenated and reduced blood.

REFERENCES

Drastich, L., 1928. *Pflüger's Arch.*, **219**: 227.

Marimoto, Takata and Sudzuki, M., 1921. *Tohoku Jour. Exper. Med.*, **2**: 258.

Sudzuki, M., 1924. *Tohoku Jour. Exper. Med.*, **5**: 419.

THE RESPIRATORY FUNCTION OF THE BLOOD OF MARINE FISHES

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INTRODUCTION

The material embodied in this paper is a report of a study of marine fish blood from the standpoint of respiratory function. Since we are now fairly cognizant of the rôle of blood in mammals, it seemed to the author that the scope of investigation should be widened by a study of species other than mammals. In choosing marine fishes as experimental material the writer had not only this point in mind, but, in addition, the thought that fishes might present some new and interesting aspect in blood physiology because of the fact that their method of blood aëration is quite different from that of mammals. The blood of mammals is apparently adjusted to the environment offered by the alveoli of the lungs where high carbon dioxide tensions prevail and oxygen tensions lower than in air exist. On the other hand, the gill of a fish is bathed in a medium where higher oxygen tensions and much lower carbon dioxide tensions prevail than is the case in the lung of a mammal. In addition to these interesting differences, fish bloods possess nucleated, instead of non-nucleated, red corpuscles, variable quantities of hemoglobin (Hall and Gray, 1929), and function in varying, rather than constant, temperatures.

There is little work on the respiratory function of fish blood to be found in the literature. Trendelenburg (1912), Gaarder (1918), Krogh and Leitch (1919), Nicloux (1923), and Wastl (1928) have investigated the blood of fishes. Krogh and Leitch found a distinct difference between oxygen dissociation curves for the bloods of the fresh-water fishes, carp, pike, and eel, and the marine cod and plaice. According to them, the hemoglobin of both types of fishes is very sensitive to carbon dioxide, and the characteristics of their blood, as far as the transportation of oxygen is concerned, are adjusted to the environment in which the fishes are living. Wastl has published oxygen dissociation curves, carbon dioxide absorption curves, and figures for arterial gas content and hydrogen ion concentration of carp blood. Distinct differences were found between the blood of the carp and that of mammals. Jolyet and Regnard (1877), and Kawamoto (1929) have studied the blood of the eel. Kawamoto determined the relationship between the oxygen dissociation of the hemoglobin and temperature. Collip (1920), Powers (1922), Jobes and Jewell (1927), and Kokubo (1927, 1930) have investigated the alkaline reserve of several fishes. Hall and collaborators (1926, 1928, 1929) have published data for the hemoglobin concentration of the blood of a number of marine species.

The investigation to be reported in this paper has been restricted for the most part to determinations of the oxygen capacities, oxygen dissociation curves, carbon dioxide absorption curves, the effect of carbon dioxide on the oxygen capacity, and the buffering capacities of the bloods. The general results have been compared with similar results obtained by other investigators on other vertebrates. The experimental work was carried on at Woods Hole, Massachusetts, in the laboratory of the United States Bureau of Fisheries.

Methods

Experimental Animals.—The fishes that were employed in the study are species common to the region of Woods Hole, Massachusetts. Three species furnished most of the results, namely, the toadfish, *Opsanus tau* (Linnaeus), the sea robin, *Prionotus carolinus* (Linnaeus), and the common mackerel, *Scomber scombrus* (Linnaeus). Some work was also done on the goosefish, *Lophius piscatorius* (Linnaeus), the scup, *Stenotomus chrysops* (Linnaeus), and the puffer, *Spheroides maculatus* (Bloch and Schneider). The fishes were maintained at the laboratory under conditions as nearly normal as possible by keeping them in "live-cars" or in hatching-boxes where plenty of running sea-water was supplied at all times. The importance of keeping them in good condition has been aptly pointed out by Hall, Gray, and Lepkovsky (1926).

The choice of the three fishes, the toadfish, sea robin, and mackerel requires some explanation. Hall and Gray (1929), and Gray and Hall (1930) have made a study of the blood sugar, hemoglobin, and iron of these fishes and found a fairly precise correlation between these factors and the activity of the fishes. The mackerel, for example, is an active fish and is characterized by a high concentration of sugar, iron, and hemoglobin in its blood, while the toadfish is a sluggish fish and is characterized by a low concentration of blood sugar, hemoglobin, and iron. The sea robin is more or less intermediate in this respect. On the basis of this information it seemed worthwhile to broaden the study enough to include several "type" fishes, instead of restricting observations to only one type. Another factor of a more practical turn was influential in the choice of these fishes. The blood of fishes does not lend itself easily to gas analysis. This has been recognized by others, and is probably one reason why more work has not been done. On account of the small size of many fishes, blood is not easily obtained for study. Some fishes have very fragile red corpuscles which makes it almost impossible to subject their blood to the drastic treatment necessary in determining dissociation curves. Also fish blood reacts peculiarly toward the reagent, potassium ferricyanide, used to liberate oxygen. As soon as the reagent comes in contact with the blood a coagulum is formed. Under these conditions it is quite impossible to liberate all the oxygen from the blood without subjecting it to vigorous, prolonged shaking. The blood from the fishes employed reacts no differently from other fish bloods toward ferricyanide, but is quite suitable in other respects. This is especially true of toadfish and sea robin bloods. Mackerel blood is quite viscous and makes pipetting rather annoying. It is also the hardest of the three to handle in the Van Slyke extraction chamber, for its coagulum adheres to the walls and is not easily cleaned out.

Obtaining of Blood Samples.—In obtaining blood for analysis an attempt was made to standardize conditions as much as possible. When it was not desired to know the gas content actually existing in the blood at the time of drawing, the procedure was to remove a fish quickly from the water and bleed it from the gills by means of a hypodermic needle attached to a 5 or 10 cc. syringe. Lithium oxalate was used as an anticoagulant. The time of bleeding was made as short as possible in order to avoid getting blood that might have excess acid in it on account of asphyxial conditions. Hall (1928) has shown that asphyxia in fishes lowers the oxygen capacity of their blood considerably.

Since most of the fishes used were small, it was found necessary to combine the blood of several specimens of a species. This practice led to no ill effects. In fact, the analytical results on different blood specimens checked more closely than otherwise on account of the averaging effect of such a procedure.

The blood was used as soon as it was drawn. In preliminary work addition of both sodium fluoride and potassium cyanide to the blood to prevent respiration of the cells and loss in carbon dioxide-combining power was tried. The results were unsatisfactory. The slight loss in carbon dioxide-combining power over a period of time did not appear to be checked. Rather than add more extraneous chemical factors, it was finally decided to modify the procedure in such a way as to avoid any appreciable error due to the activity of the cells. This necessitated using a given sample of blood a shorter length of time and checking a curve that had once been established by means of freshly drawn blood.

It also made it necessary that a blood sample be analyzed for its gas content as soon as it had come into equilibrium with a given gas tension, and that the gas phase be separated from the blood remaining in the tonometer during the time consumed in the analysis. It should be mentioned at this time that Dr. F. G. Hall (unpublished) has determined the oxygen consumption of these bloods and shown, under the conditions of the author's technique, that the error arising from oxygen consumption of the cells would be negligible over the short period of time that elapses in getting a blood sample into the Van Slyke apparatus from the tonometer.

When it was desired to know the actual content of gases existing in the blood at the time of drawing, the method was modified to suit the purpose. In attempts to determine arterial or venous gas contents, fishes were placed in suitable traps and a stream of fresh sea-water directed over their gills. The blood was then drawn under oil and the gases immediately analyzed. It is most difficult to get a satisfactory technique for determining arterial and venous gases in fishes. The results obtained are only approximate at best.

Determination of Erythrocyte Count and Volume.—The number of red corpuscles per cubic millimeter of blood was determined by employing the usual procedure. The volume of red corpuscles was determined by an haematocrit especially designed by Dr. F. G. Hall for use with fish blood.

Equilibration of Blood with Gases and Determination of Gases.—The gases used in these experiments were carbon dioxide, oxygen, and nitrogen. The required mixtures were made in a mixing chamber attached to an ordinary gas burette (if gas mixtures different from air were desired). The method of handling the blood and gases was essentially the same as that prescribed by Austin *et al.* (1922), except for the admittance of gases to tonometers. Instead of using the method they prescribe, the tonometers were filled with clean, neutral mercury, and the gas mixtures drawn into them from the mixing chamber by withdrawing the mercury. The equilibration of blood samples was carried out according to their "first saturation method," using the double tonometer. Equilibration for all samples was allowed to take place at 20° C. and at atmospheric pressure. Atmospheric pressure was maintained by occasionally opening the stop-cock on the tonometer. Since the gases in the tonometer were always analyzed after equilibration, the entrance of a small amount of gas from the atmosphere did no harm. The tonometers were mechanically rotated in a thermostatically controlled water bath for a period of about 30 minutes. It was found in preliminary experiments that this was sufficient time to allow the blood and gas phase to come into equilibrium with each other. Usually one tonometer was rotated at a time. However, in some of the work involving carbon dioxide absorption, two tonometers were used simultaneously, one containing reduced and the other oxygenated blood.

At the end of equilibration a sample of blood was removed from the tonometer and the gases in it immediately analyzed according to the technique of Van Slyke and Neill (1924). Both oxygen and carbon dioxide were simultaneously liberated from the blood by using acid ferricyanide. One cubic milliliter of blood was used for each analysis, and was admitted to the extraction chamber by means of a Van Slyke differential pipette. The blood was agitated in the extraction chamber a little longer than is usual for mammalian blood. This was found necessary in order to insure the complete liberation of gases. Both the carbon dioxide and oxygen were absorbed after liberation, sodium hydroxide being used for carbon dioxide, and sodium hydrosulfide for oxygen.

TABLE I

Oxygen capacity determinations. Blood equilibrated with air at 20° C.

Species	Oxygen capacity	Red Blood Corpuscles	Hæmatocrit	Iron
	vol. per cent	*cu. mm.*	*vol. per cent*	*mg. 100 cc.*
Goosefish	5.07	867,083	15.45	13.40
Toadfish	6.21	585,000	19.50	14.00
Puffer	6.75	2,284,000	17.50	21.50
Scup	7.30	2,685,000	32.60	24.60
Sea robin	7.66	2,536,000	24.00	23.10
Mackerel	15.77	3,000,000	37.10	37.10

The amount of carbon dioxide and oxygen in blood was expressed as volumes per cent of dry gas at 760 mm., and 0° C., the tables prepared by Van Slyke and Neill (1924) being used for oxygen, and those prepared by Van Slyke and Sendroy (1927) for carbon dioxide. In determining the oxygen combined with hemoglobin, the amount of oxygen physically dissolved was calculated on the basis of Bohr's (1905) solubility coefficients. A special equation similar to that of Peters, Bulger, and Eisenman (1923) was employed in the calculation to allow for the variable corpuscular volume in the various bloods.

The concentration of the gaseous phase in the tonometers was determined after equilibration of blood samples by analysis in the Haldane apparatus as modified by Henderson (1918). The results were expressed in terms of tension by employing the usual calculations.

Method of Studying Lactic Acid Effect.—Lactic acid was carefully added to small samples of blood in amounts necessary to give the desired concentration. The blood was then equilibrated in air and handled the same as in the other experiments.

Calculation of pH of Blood.—In calculating the pH of fish blood the familiar Henderson-Hasselbalch equation was used (Henderson, 1908; Hasselbalch, 1917). A pK′ factor of 6.24 was employed for the blood at 20° C. This was derived by using the average pK′ factor of 6.13 that has been worked out for mammalian serum at 38° C. (using Bohr's, 1905, solubility coefficient for CO_2) by a series of workers (Warburg, 1922; Cullen, Keeler, and Robinson, 1925; Van Slyke, Hastings, Murray, and Sendroy, 1925; and Hastings, Sendroy and Van Slyke, 1928), and adding a temperature correction of 0.005 for each degree below 38° C. (Hasselbalch, 1917; and Warburg, 1922). In addition a correction of 0.02 was added because whole blood was used instead of serum. The pK′ factor for whole blood is slightly higher than that for serum (Warburg, 1922; Peters, Bulger, and Eisenman, 1923; and Van Slyke *et al.*, 1925).

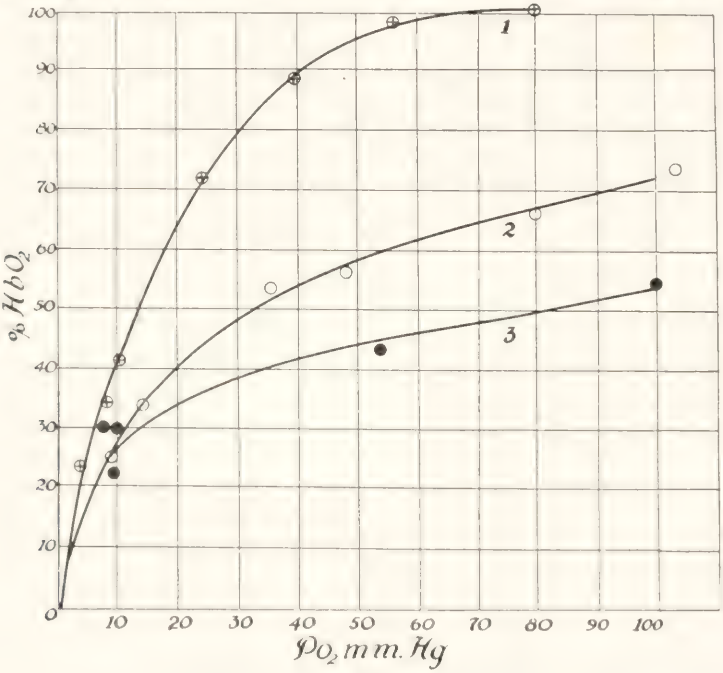

FIG. 1. Oxygen dissociation curves for toadfish blood at 20° C. Curve 1 at 1 mm. carbon dioxide; curve 2 at 10 mm. carbon dioxide; and curve 3 at 25 mm. carbon dioxide tension.

In using the pK′ factor in the following calculations of pH, it is recognized that there are many variables which enter into its composition for any one blood, especially when it is applied to whole blood. Warburg (1922), Hastings and Sendroy (1925), Stadie and Hawes (1928), and Stadie (1928) have shown that the pK′ factor is affected by the ionic strength of the solution in which it is measured. Furthermore, the researches of Warburg (1922), Van Slyke, Wu and McLean (1923), and Peters, Bulger, and Eisenman (1923) have demonstrated the effect of degree of oxygenation of blood, its pH, and its relative volume of corpuscles and plasma upon the pK′ factor.

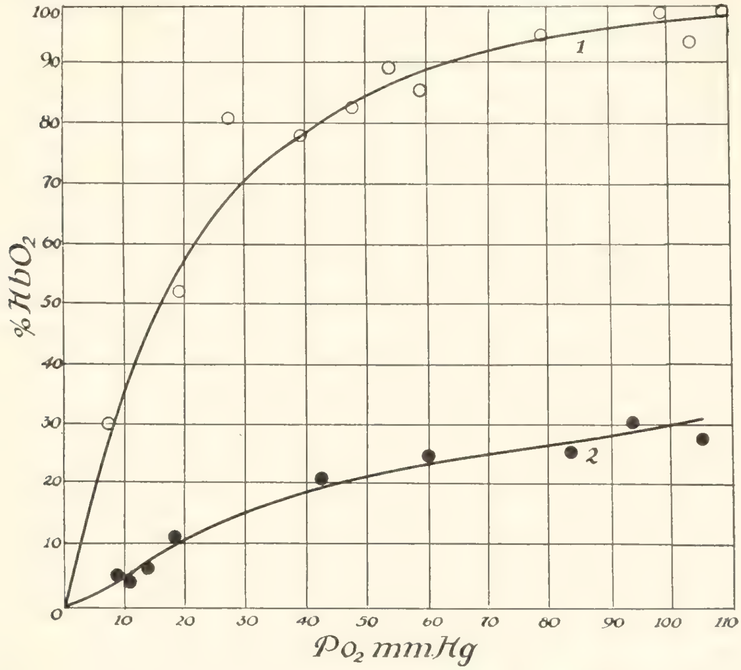

FIG. 2. Oxygen dissociation curves for sea robin blood at 20° C. Curve 1 at 1 mm. carbon dioxide; and curve 2 at 25 mm. carbon dioxide tension.

However, there is little information at the present time that will permit the calculation of the pH of fish blood with the degree of refinement that now seems possible for mammalian blood. Therefore the author does not claim absolute accuracy for the calculated pH of fish blood, but only relative, and admits that with the advent of more information his figures will probably require correction.

RESULTS

A. The Transportation of Oxygen

Oxygen Capacity of Blood.—The results of this study are summarized in Table I. The figures for oxygen capacity are those obtained when the blood was equilibrated in air, and dissolved oxygen subtracted. Thus they represent the actual amount of oxygen combined with hemoglobin under the conditions of the experiment. An attempt has been made to correlate oxygen capacities of the various bloods with their corpuscle count, corpuscle volume, and iron content. The author is indebted to Dr. F. G. Hall and Mr. S. R. Tipton for some of the data contained in the last three columns of Table I. It should be mentioned that the figures for corpuscle count, corpuscle volume, and iron content were not always obtained from the same samples of blood on which oxygen capacity determinations were made. The data represent the average of a considerable number of determinations. There appears to be a general correlation between the oxygen capacity of fish blood and the previously mentioned factors. The best agreement exists between iron and oxygen. Since the corpuscle count and volume are variable among themselves, on account of differences in size of corpuscles, these factors do not show as good a correlation as iron.

TABLE II

Gases in Blood, as Drawn under Oil

Species	Kind of Blood	CO_2	O_2	P_{CO_2}	P_{O_2}	HbO_2	Conditions of Drawing
		vol. per cent	*vol. per cent*	*mm. Hg.*	*mm. Hg.*	*per cent*	
Scup	Arterial	8.15	8.33	—	—	—	Water over gills Blood from gills
		9.16	5.00	—	—	59	Water over gills Blood from caudal artery
		8.90	5.24	—	—	69	Water over gills Blood from gills
		11.40	3.13	—	—	—	Water over gills Blood from gills
Sea robin		6.15	2.55	2	10	33.2	Water over gills Blood from gills
Toadfish	Venous	13.30	0.54	10	2	7.6	Water over gills Blood from heart
Goosefish		10.25	Trace	—	—	—	Water over gills Blood from bulbus
Puffer		14.90	0.34	—	—	5.3	Water over gills Blood from sinus venosus
Sea robin	Asphyxial	9.08	2.41	4	20	31.5	Fish in air Blood from gills
		13.40	1.59	10	20	22.2	Fish in air Blood from gills

The most interesting feature of this phase of the work is that it points out great differences in the oxygen capacities of the various bloods. The sluggish goosefish and toadfish possess bloods of low oxygen capacity, whereas the active mackerel has a blood of high oxygen capacity.

TABLE III

Oxygen dissociation of blood. Equilibrated at 20° C.

Species	P_{CO_2}	P_{O_2}	O_2-Capacity	O_2-Content	O_2-Dissolved	O_2-Combined	HbO_2	pH
	mm. Hg	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	
Toadfish	0.762	3.75	6.84	1.56	0.015	1.54	22.5	7.86
	0.454	7.80	5.13	1.75	0.030	1.72	33.5	7.99
	1.150	10.30	6.31	2.66	0.040	2.62	41.6	7.68
	0.615	24.60	5.13	3.78	0.096	3.68	71.8	7.60
	0.765	39.20	5.13	4.70	0.152	4.55	88.3	7.78
	0.690	56.20	5.13	5.28	0.219	5.06	98.6	7.70
	0.690	80.00	5.13	5.51	0.312	5.20	101.4	7.66
	8.62	8.85	6.31	1.65	0.035	1.62	25.6	7.33
	10.85	13.35	6.68	2.37	0.052	2.32	34.6	7.21
	11.15	35.70	6.68	3.64	0.138	3.50	52.3	7.18
	11.25	48.20	6.68	4.00	0.188	3.81	57.0	7.16
	10.42	80.00	6.68	4.75	0.312	4.44	66.4	7.17
	10.28	103.00	6.68	5.25	0.400	4.85	72.6	7.16
	25.40	7.47	5.56	1.63	0.029	1.60	28.8	6.98
	25.10	9.63	6.31	1.43	0.037	1.37	22.0	7.00
	27.40	10.00	6.84	1.93	0.039	1.89	27.5	6.94
	25.80	53.00	5.56	2.62	0.207	2.41	43.5	6.97
	25.05	100.00	5.56	3.38	0.390	2.99	53.8	6.98
Sea robin	0.304	6.69	7.02	2.15	0.026	2.12	30.2	8.22
	0.485	19.40	7.80	4.09	0.075	4.02	52.1	8.16
	0.227	27.00	8.20	6.72	0.100	6.62	80.7	8.03
	0.727	39.70	6.82	5.47	0.155	5.32	78.0	7.86
	1.050	47.20	7.91	6.70	0.184	6.52	82.5	7.67
	0.455	54.50	8.20	7.55	0.212	7.34	89.5	7.67
	1.510	59.70	7.25	6.40	0.233	6.17	85.1	7.68
	1.160	79.00	7.25	7.22	0.308	6.91	95.3	7.43
	1.132	99.00	7.91	8.19	0.386	7.80	98.6	7.13
	0.761	104.50	7.04	6.94	0.408	6.53	93.0	7.83
	0.225	109.00	7.20	7.50	0.425	7.08	98.3	8.09
	24.70	9.35	7.66	0.42	0.036	0.38	6.3	7.05
	28.10	10.75	7.10	0.32	0.042	0.28	4.0	6.98
	25.00	13.50	6.85	0.50	0.053	0.45	6.5	7.03
	26.10	17.00	7.15	0.85	0.066	0.78	11.0	7.00
	24.40	17.80	6.97	0.89	0.069	0.82	11.6	7.04
	21.80	18.20	7.15	0.83	0.071	0.76	10.8	7.13
	25.10	42.00	7.15	1.61	0.164	1.45	20.2	7.06
	25.60	60.50	7.15	1.94	0.236	1.70	23.8	7.04
	26.00	84.50	6.97	2.12	0.330	1.79	25.6	7.05
	25.90	92.50	7.15	2.50	0.360	2.14	30.0	7.00
	23.60	106.50	6.97	2.36	0.415	1.95	28.0	7.12

TABLE III—*Continued*

	P_{CO_2}	P_{O_2}	O_2-Capacity	O_2-Content	O_2-Dissolved	O_2 Combined	HbO_2	pH
	mm. Hg	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	
Mackerel	1.130	4.74	16.41	1.76	0.018	1.74	11.0	8.19
	1.250	8.30	15.76	3.46	0.032	3.43	21.6	8.00
	0.640	17.70	14.72	8.11	0.067	8.04	53.9	8.17
	0.977	31.95	16.29	12.45	0.122	12.33	75.8	7.96
	0.754	45.00	16.64	13.60	0.172	13.43	80.7	8.03
	0.382	64.60	17.81	16.35	0.247	16.10	90.43	8.00
	0.825	75.60	16.64	15.30	0.290	15.01	90.2	7.86
	0.768	98.70	15.59	14.75	0.378	14.37	92.2	7.48
	0.758	115.00	16.64	16.10	0.440	15.66	94.1	7.60
	10.17	9.87	14.54	1.01	0.038	0.97	6.7	7.49
	10.10	14.50	14.54	2.15	0.055	2.10	14.5	7.58
	11.30	29.40	14.54	4.13	0.113	4.02	27.7	7.51
	10.45	46.80	14.54	7.15	0.179	6.97	48.0	7.51
	11.00	65.00	14.54	7.70	0.249	7.45	52.0	7.49
	10.30	77.50	14.54	9.82	0.297	9.52	65.5	7.43
	10.35	83.30	14.54	10.30	0.319	9.98	68.6	7.46
	10.85	101.00	14.54	11.00	0.387	10.61	73.0	7.42
	24.50	12.00	17.81	0.92	0.046	0.87	5.0	7.33
	18.85	40.50	16.62	6.35	0.155	6.20	37.3	7.36
	24.80	53.40	16.62	7.25	0.204	7.05	42.5	7.25
	24.80	69.50	16.62	8.65	0.267	8.38	50.5	7.16
	25.40	70.30	16.62	9.50	0.269	9.23	55.6	7.14
	24.30	90.00	16.62	11.08	0.345	10.74	64.6	7.14
	19.60	101.50	17.81	9.58	0.389	9.19	51.7	7.33
	23.90	115.20	16.62	12.70	0.442	12.26	73.8	7.11

Oxygen Content of Blood.—Any attempt to determine the actual amount of gas existing in the arterial or venous blood of fishes as small as those used in this investigation is beset with difficulties. The few results obtained are recorded in Table II. Attempts to get arterial blood from these fishes were rewarded with little success. Analysis of blood removed from efferent gill arteries of the scup and sea robin showed much less oxygen than could reasonably be expected. It would appear that the syringe used in the operation hastened the circulation through the gill to a point where the blood had not sufficient time to become aërated to the normal degree. A more reliable source of arterial blood is that from the caudal artery, but the fishes used are unsuited for getting blood from such a source. Until a more adequate technique is devised, any statement as to the actual oxygen content of arterial blood in these fishes will have to be postponed. Hall (1930) reported 85 per cent oxygen saturation in mackerel arterial blood. Wastl (1928) found 93 per cent oxygen saturation in carp blood.

With regard to the oxygen content of venous blood, more satisfactory results were obtained. Practically no oxygen was found in the venous blood of the goosefish, toadfish, and puffer.

The gas tensions recorded in Table II for sea robin and toadfish bloods were not determined experimentally but were interpolated from the oxygen dissociation curves for their bloods.

Oxygen Dissociation of Hemoglobin.—Table III, and Figs. 1, 2, and 3 summarize the results of this study. At a carbon dioxide tension of approximately one millimeter toadfish hemoglobin is characterized by a steeper dissociation curve than either sea robin or mackerel. The hemoglobins of the latter appear to act quite the same toward oxygen, at this carbon dioxide tension, except for the fact that sea robin hemoglobin tends to become saturated a little more quickly than mackerel at the higher oxygen tensions. At 10 mm. carbon dioxide tension the dissociation curves for mackerel and toadfish hemoglobins are flattened most remarkably. A still more pronounced flattening is produced at 25 mm. carbon dioxide tension. Of the three hemoglobins the sea

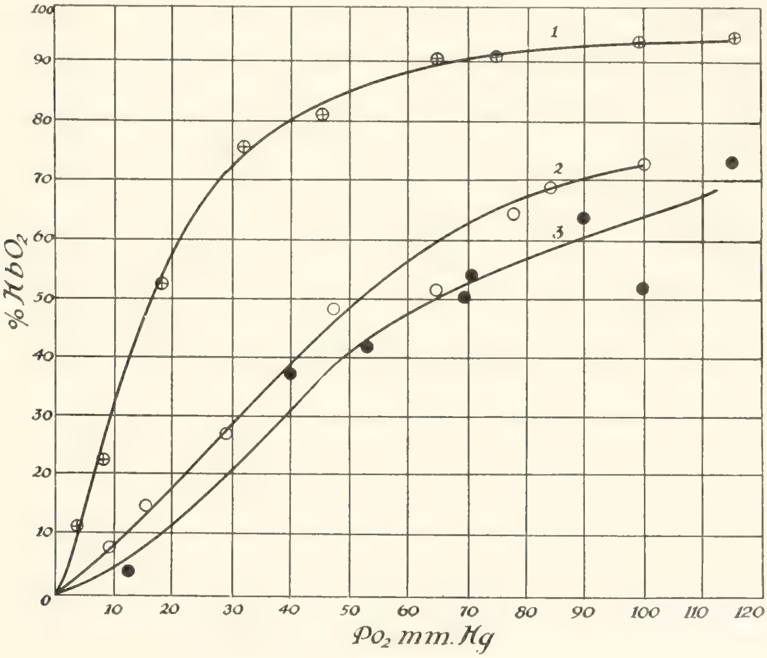

FIG. 3. Oxygen dissociation curves for mackerel blood at 20° C. Curve 1 at 1 mm. carbon dioxide; curve 2 at 10 mm. carbon dioxide; and curve 3 at 25 mm. carbon dioxide tension.

robin's is most affected at the latter carbon dioxide tension. The appearance of the curves at 10 and 25 mm. of carbon dioxide is very interesting. There is a tendency for them to become nearly asymptotic with respect to the abscissa before saturation is complete. This is most noticeable in the curves for toadfish and sea robin hemoglobins. At 10 mm. carbon dioxide tension, in the case of toadfish hemoglobin, the curve appears to approach a limit at approximately 75 per cent oxygen saturation. At 25 mm. carbon dioxide this same tendency occurs in sea robin hemoglobin at 25 per cent saturation, while toadfish hemoglobin shows this at about 50 per cent saturation. The curves for mackerel hemoglobin do not show any very marked tendency to become asymptotic. In the case of toadfish and sea robin bloods it would appear as if carbon dioxide affected not only the oxygen dissociation constant of the hemoglobin, but, also, that the quantity of oxygen with which the hemoglobin can combine is reduced by the presence of carbon dioxide. Redfield and Mason (1928) have pointed out that such an effect is produced by acid in the case of purified *Limulus* hemocyanin.

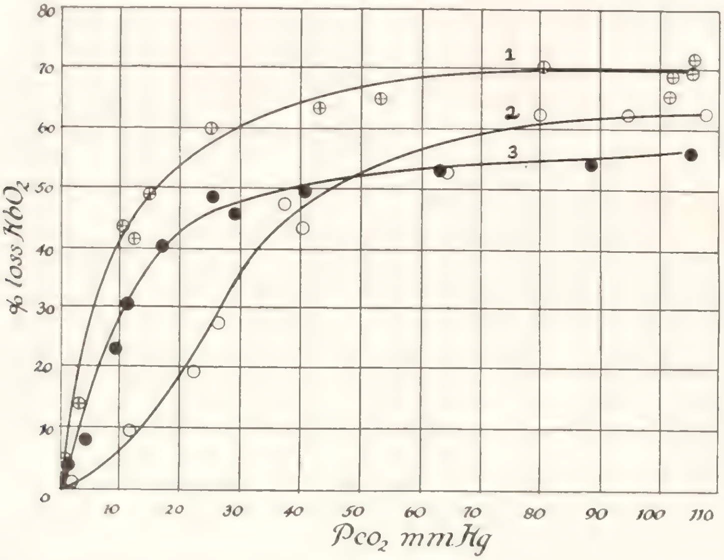

FIG. 4. Effect of carbon dioxide on the oxygen capacity at 20° C. Curve 1 is for sea robin blood; curve 2 for mackerel blood; and curve 3 for toadfish blood.

Effect of Carbon Dioxide on the Oxygen Capacity.—The peculiar effects of carbon dioxide on the oxygen dissociation curves suggested an investigation of its effect on the so-called oxygen capacity. For this work blood samples were equilibrated with 153 mm. of oxygen and varying tensions of carbon dioxide. The results are presented in Table IV, and Figs. 4 and 5. As can be seen from the data, carbon

TABLE IV

Effect of carbon dioxide on oxygen capacity. Blood equilibrated at constant P_{O_2}(152 mm.) at 20° C.

Species	P_{CO_2}	O_2-Capacity	O_2-Content	O_2 Dissolved	O_2 Combined	Loss in oxygen capacity	pH
	mm. Hg	*vol. per cent at O* P_{CO_2}	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	
Toadfish	1.37	6.87	7.22	0.60	6.62	3.64	7.64
	1.54	6.34	6.67		6.07	4.27	7.52
	4.62	6.34	6.50		5.90	6.94	7.42
	9.15	6.34	5.50		4.90	22.70	7.23
	11.75	6.40	5.03		4.43	30.80	7.20
	16.70	6.34	4.38		3.78	40.40	7.08
	25.70	6.34	3.85		3.25	48.80	6.98
	29.30	5.59	3.60		3.00	46.40	6.94
	41.00	6.63	3.98		3.38	49.20	6.82
	63.70	6.40	3.57		2.97	53.60	6.71
	88.00	6.40	3.50		2.90	54.70	6.60
	106.00	6.87	3.58		2.98	56.60	6.48
Sea robin	1.21	7.67	7.92	0.60	7.32	4.50	7.79
	3.79	7.67	7.15		6.55	14.60	7.56
	10.50	7.71	4.95		4.35	43.60	7.30
	12.15	7.67	5.06		4.46	41.80	7.27
	15.25	7.71	4.53		3.93	49.00	7.11
	25.05	7.69	3.67		3.07	60.00	7.06
	43.60	7.15	3.20		2.60	63.60	6.83
	53.00	7.67	3.26		2.66	65.40	6.75
	80.80	8.29	3.04		2.44	70.60	6.68
	102.00	7.15	3.06		2.46	65.60	6.60
	103.00	8.70	3.34		2.74	68.60	6.58
	106.50	7.15	2.82		2.22	69.00	6.57
	107.00	8.15	2.90		2.30	71.80	6.56
Mackerel	2.17	16.43	16.80	0.586	16.21	1.34	7.94
	2.26	16.78	17.15		16.56	1.31	7.84
	12.00	15.64	14.75		14.16	9.90	7.37
	22.50	16.64	14.05		13.46	19.10	7.08
	26.10	14.51	11.05		10.46	27.90	7.21
	37.30	16.78	9.40		8.81	47.50	7.10
	40.80	16.43	9.81		9.22	43.80	7.16
	65.05	16.60	8.35		7.76	53.25	6.95
	80.00	16.78	6.90		6.31	62.60	6.88
	95.00	16.78	7.06		6.47	61.60	6.84
	108.50	16.43	6.96		6.37	61.20	6.82

dioxide affects a very marked loss in oxygen-combining power of the hemoglobins. However, a maximum loss is reached beyond which further addition of carbon dioxide has little or no effect. Sea robin hemoglobin suffers the greatest loss in oxygen-combining power, the maximum being around 70 per cent, whereas the maximum for toadfish is about 55 per cent. Mackerel hemoglobin has a maximum loss between those for the other two. The data procured seem to corroborate what was already anticipated in a study of the dissociation curves, namely that the ability of the hemoglobins to combine with oxygen is greatly reduced in the presence of carbon dioxide.

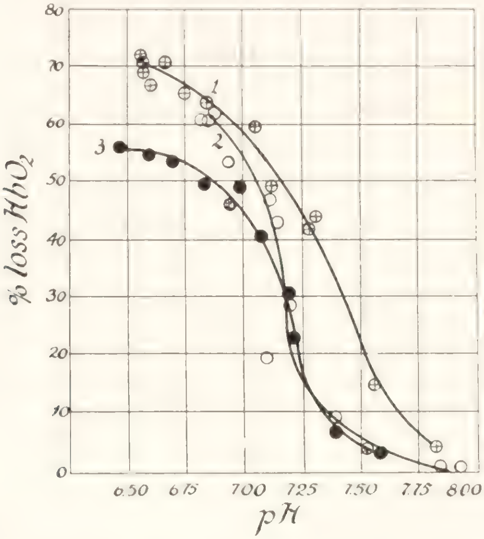

FIG. 5. Effect of pH on the oxygen capacity at 20° C. Curve 1 is for sea robin blood; curve 2 for mackerel blood; and curve 3 for toadfish blood.

Plotting loss in oxygen capacity, or, as designated in Fig. 5, loss in oxyhemoglobin, as a function of pH, yields sigmoid curves for the three hemoglobins. Within a certain range of pH there is a marked loss in oxygen-combining power. Outside this range at either end, within the limits of pH established in these experiments, loss in oxygen-combining power is relatively slight.

Effect of Lactic Acid on Oxygen Capacity.—It was thought advisable to modify the pH of the bloods by other means than the use of carbon dioxide and see if a similar effect on the oxygen capacity could be obtained. Therefore blood samples containing definite concentrations of lactic acid were equilibrated in 153 mm. of oxygen. In this case, of course, no carbon dioxide was added to the gaseous phase in the tonometers. Only the blood of the sea robin was used in these experiments. The results are shown in Fig. 6. A greater loss of oxyhemoglobin was observed at the higher concentrations of lactic acid than was found when carbon dioxide was used, though the calculated pH was less. However, there may have been some other factor entering in to produce the results, such as the formation of methemoglobin, and, since this was not ascertained, no emphasis should be placed on the magnitude of the results. The main matter of interest is that, in general, the form of the curve is similar to that for the carbon dioxide effect.

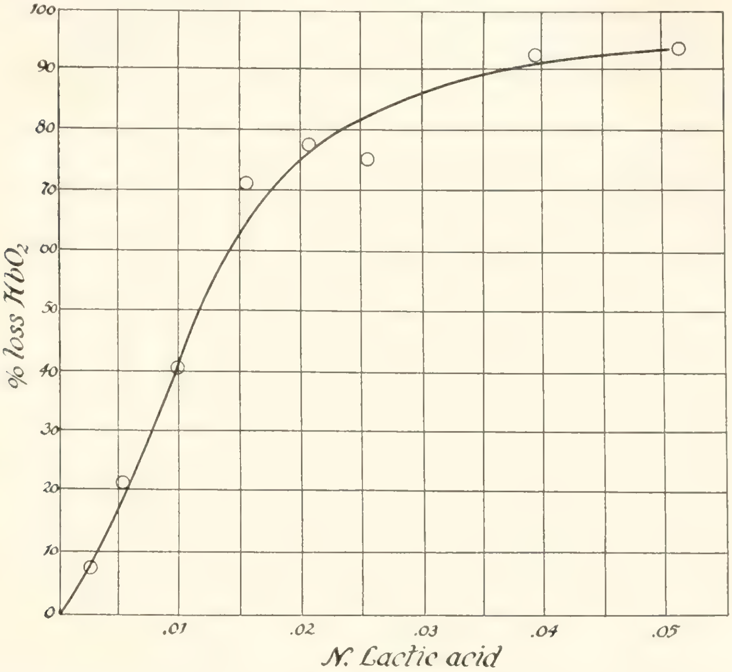

FIG. 6. Effect of lactic acid on the oxygen capacity at 20° C. Curve for sea robin blood only.

B. The Transportation of Carbon Dioxide

Carbon Dioxide Content of Blood.—An attempt was made to determine the amount of carbon dioxide normally present in the circulating blood. The results are recorded in Table II. The bloods of the fishes studied contain relatively little carbon dioxide. The tension even in the venous blood is probably not more than 10 to 15 millimeters.

Carbon Dioxide Absorption of Blood.—The results of this study are presented in Figs. 7, 8, and 9. Of the three bloods examined the toadfish was found to take up the least, and the mackerel to take up the most carbon dioxide. All three curves tend to flatten out above 10 mm. carbon dioxide tension, the flattening being most pronounced in the case of toadfish blood, and least in mackerel. The curve for mackerel blood is quite out of the class of the curves for the other two fishes. Apparently mackerel blood has a higher available base than toadfish and sea robin bloods.

Christiansen, Douglas, and Haldane (1914) were the first to discover that reduced blood will take up more carbon dioxide than oxygenated blood. This phenomenon has been explained since their work was published by the assumption that oxyhemoglobin is a stronger acid than hemoglobin, and, thus, base is liberated and made available for carbon dioxide when oxyhemoglobin is reduced. The elucidation of the fact is due mainly to the work of Van Slyke and his collaborators at the Rockefeller Institute.

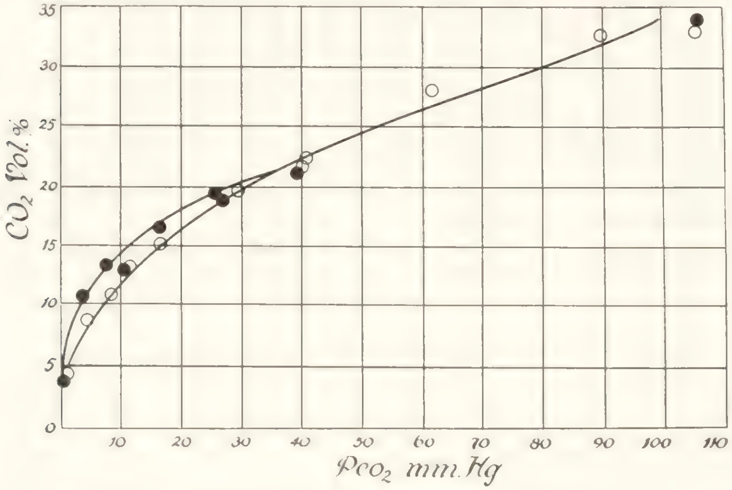

FIG. 7. Carbon dioxide-absorption curves for toadfish blood at 20° C. The dots are for reduced, and the circles for oxygenated blood.

Since it has been demonstrated beyond doubt that reduced blood will take up more carbon dioxide than oxygenated, as far as mammals are concerned, it was thought advisable to determine whether a similar phenomenon could be shown for fish blood. Wastl (1928) has shown such to be the case as far as carp blood is concerned. The results obtained on the bloods of the toadfish, sea robin, and mackerel are shown in the carbon dioxide-absorption curves drawn in Figs. 7, 8, and 9. Toadfish and sea robin bloods show little difference in the ability of reduced and oxygenated to absorb carbon dioxide. Within what appears to be the physiological range of carbon dioxide tension (from analyses of the carbon dioxide content of venous blood), however, reduced blood takes up slightly more carbon dioxide than oxygenated. With respect to mackerel blood the range where this can be demonstrated is considerably greater, and the curves begin to take on the appearance of mammalian carbon dioxide-absorption curves. There are probably at least two reasons why it is difficult to demonstrate greater carbon dioxide absorption by reduced than by oxygenated blood in the case of the first two fishes: (1) the small amount of hemoglobin present to furnish base in changing from the oxygenated to the reduced state, and (2), the effect of carbon dioxide in reducing the oxygen capacity. One can hardly say he is dealing with oxygenated blood at high carbon dioxide tensions, for under these conditions the oxygenation of the blood is greatly reduced.

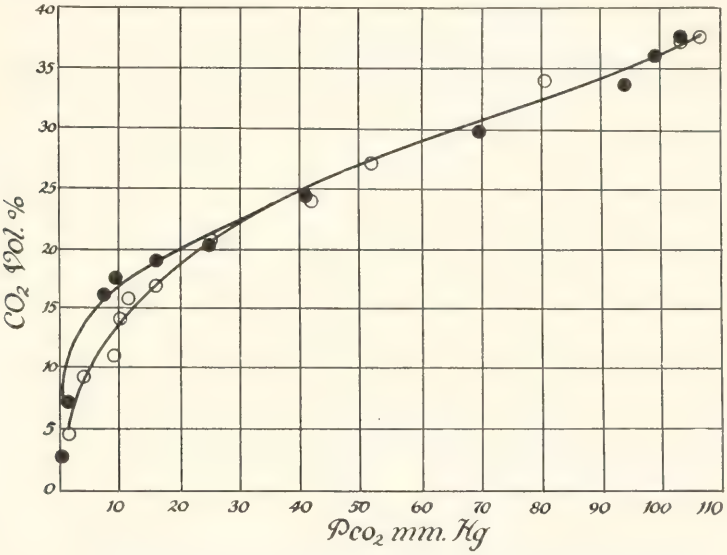

FIG. 8. Carbon dioxide-absorption curves for sea robin blood at 20° C. The dots are for reduced, and the circles for oxygenated blood.

Buffering Ability of Blood.—The $BHCO_3$ concentrations of the bloods have been calculated and the results plotted as a function of pH. Such a procedure will point out their relative buffering ability. The curves obtained are shown in Figs. 10, 11, and 12. In general, within the normal range of pH, reduced blood has a higher concentration of $BHCO_3$ at a given pH than oxygenated. This means that by reduction oxyhemoglobin imparts to the blood a certain protection against change in pH, for a certain added amount of carbon dioxide may be taken up at the same hydrogen ion concentration. Outside the normal pH range the curves for oxygenated and reduced blood tend to converge so that there is practically no difference in the ability of the two states of blood to bind carbon dioxide.

A comparison of the three bloods shows at once that mackerel blood is much better buffered than either toadfish or sea robin. Toadfish blood is buffered the least of all. However, there is little difference between it and sea robin blood.

DISCUSSION

In the work on the effect of carbon dioxide on the oxygen capacity and on the oxygen dissociation curves a suggestive series of results were obtained. In mammalian hemoglobin the usual effect of carbon dioxide is purely on the oxygen dissociation constant, a simple Bohr effect with no upset in the original oxygen capacity of the particular hemoglobin studied. The hemoglobins of these fishes, however, seem to be affected by carbon dioxide in a manner more complicated. The data suggest that some of the oxygen-binding groups of the hemoglobin molecule have become inactive. If the hemoglobin molecule combines with four molecules of oxygen, as has been suggested by Adair (1925), it would appear as if carbon dioxide were inactivating one or more of the four prosthetic groups involved in binding oxygen. In other words, it

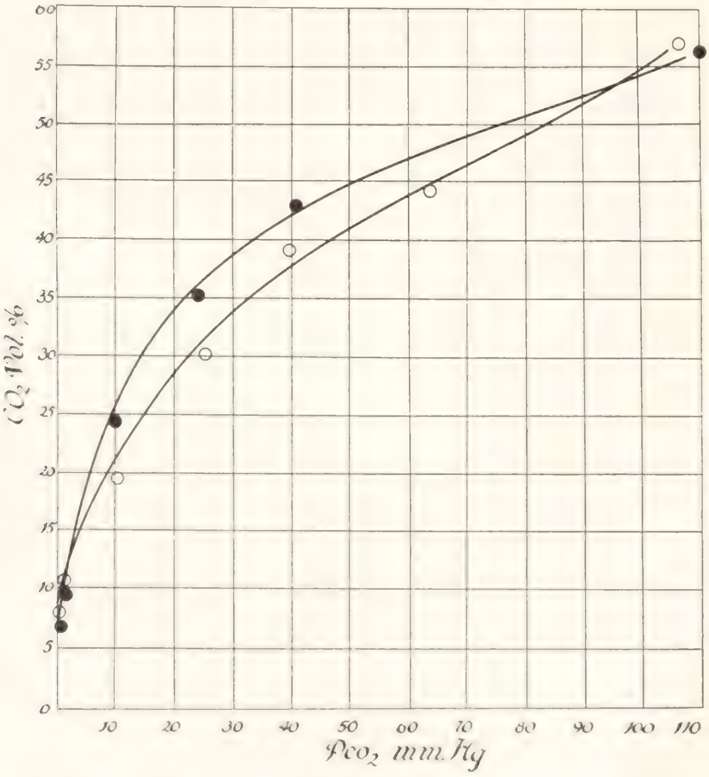

FIG. 9. Carbon dioxide-absorption curves for mackerel blood at 20° C. The dots are for reduced, and the circles for oxygenated blood.

would look as though the hemoglobin-oxygen reaction were stopping off at one or more of the intermediate compound stages, depending upon how much carbon dioxide is present, instead of the reaction being carried completely through the four theoretical steps presented by Adair.

To illustrate this point attention is recalled to the results on the direct effect of carbon dioxide on oxygen capacity. In the case of toadfish blood there is produced a maximum loss of about 55 per cent in oxygen capacity in the presence of carbon dioxide, and in sea robin blood about 70 per cent. Mackerel blood under the same conditions experiences a loss of slightly over 60 per cent. Interpreting this situation on the basis of inactivation of oxygen-binding groups, toadfish hemoglobin has two of the four groups inactivated. Thus, allowing for experimental errors, the oxygen capacity drops to a point approximately 50 per cent lower than the original figure for oxygen capacity obtained when the blood was equilibrated in air. Sea robin hemoglobin, and perhaps mackerel, has three of the four groups inactivated. Thus the new figure for oxygen capacity obtained in the presence of considerable carbon dioxide is approximately 75 per cent lower than the original. As has been pointed out previously, these marked drops in oxygen capacity occur at definite ranges of pH.

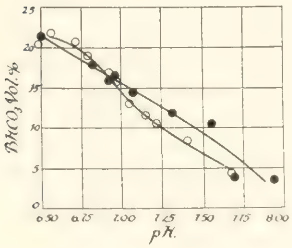

FIG. 10.

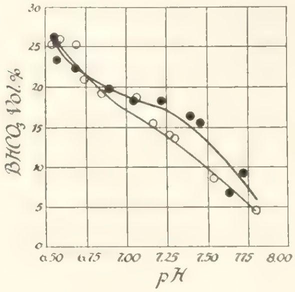

FIG. 11.

FIG. 10. $BHCO_3$: pH curves for toadfish blood at 20° C. The dots are for reduced, and the circles for oxygenated blood.

FIG. 11. $BHCO_3$: pH curves for sea robin blood at 20° C. The dots are for reduced, and the circles for oxygenated blood.

It will be recalled that reference was made to the peculiar tendency of the oxygen dissociation curves (most marked in the case of those for the toadfish and sea robin) to appear to reach a limit considerably before the 100 per cent oxygen-saturation point was reached. It seems reasonable to suppose that the phenomenon of inactivation of oxygen-binding groups affords an interpretation of this situation.

There is no doubt but that there is danger in carrying the foregoing interpretation too far. The author wishes to emphasize the fact that the idea of inactivity brought forth in this paper is purely suggestive. Data are far too few to warrant any definite conclusion. If the data really mean that certain prosthetic groups are inactivated, then the oxygen-dissociation curves should present asymptotic relationships from the point of minimum oxygen tension at which the remaining active groups are saturated with oxygen up through oxygen tensions far above those used in these experiments. At the same time the same marked loss in oxygen capacity in the presence of carbon dioxide should be capable of demonstration even though the blood were equilibrated in pure oxygen. It is regretted that higher oxygen tensions were not used, for it seems that if such had been the case the idea of inactivity would have had either a stronger case in its favor or been thrown out entirely. It may be that the entire situation is a greatly exaggerated Bohr effect, and all that is necessary is higher oxygen tensions to bring back the original oxygen capacity.

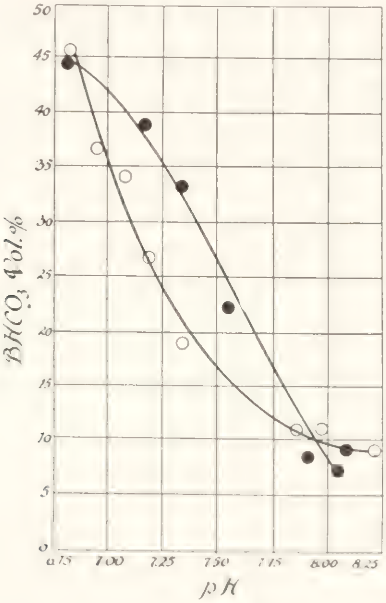

FIG. 12. $BHCO_3$: pH curves for mackerel blood at 20° C. The dots are for reduced, and the circles for oxygenated blood.

If the idea presented in this paper proves upon further experimentation to be correct, we have before us a means of furthering the study of Adair's theory of the combination of oxygen with hemoglobin.

Aside from the physical chemistry of fish hemoglobin, the relation of the data presented to the life of the fish is interesting. We find a correlation between the transportation of oxygen and the environment and habits of the fishes. The sluggish fishes have bloods of low oxygen capacity, and the active of high capacity. Thus, there is evidence of adjustment between oxygen capacities and oxygen requirements, for, as Hall (1929) has shown, the sluggish fishes do not consume as much oxygen per unit time as the active. Further evidence of adjustment is shown in the form of the oxygen dissociation curves at low carbon dioxide tensions. The toadfish hemoglobin, under these conditions, becomes saturated with oxygen at a much lower tension than is the case with the other two fishes. This may partially explain the ability of this fish to live in water of abnormally low oxygen tension (Hall, 1930). On the other hand, mackerel hemoglobin, in the presence of 1 mm. of carbon dioxide, requires a considerably greater tension of oxygen to become saturated than is the case for the other fishes studied. This may account in part for the great susceptibility of the mackerel to asphyxiation. Hall (1930) found that a mackerel requires a strong circulation of oxygen-loaded sea water over its gills in order to prevent excessive oxygen-unsaturation of its blood, and consequent death due to asphyxia.

The high sensitivity of all three hemoglobins to carbon dioxide indicates that they are adjusted to an environment of low carbon dioxide tension, such as the gills offer. Any one of the fishes examined would experience considerable difficulty in getting sufficient oxygen were the environment in which its gills are bathed loaded with free carbon dioxide. Krogh and Leitch (1919) and Redfield *et al.* (1926) have alluded to the apparent adjustment of the oxygen dissociation curves to the environment and habits of animals. Krogh and Leitch offered such a conclusion after working on the blood of fishes, while Redfield and collaborators came to the same conclusion after investigating certain bloods containing hemocyanin. The work presented here corroborates their evidence.

With regard to the transportation of carbon dioxide by the blood of marine fishes, this investigation shows that the amount bound by the various bloods is not the same for all species. Directionally the same differences occur as were found in the ability of the bloods to combine with oxygen. Mackerel blood is not only able to bind greater quantities of oxygen, but is also able to bind greater quantities of carbon dioxide than either toadfish or sea robin blood. This strongly suggests that the greater concentration of hemoglobin in mackerel blood is responsible for the difference noted. It is known that hemoglobin affects the height and slope of carbon dioxide-absorption curves. This has been pointed out by Peters, Bulger, and Eisenman (1924) and others. The writer, too, found that anaemic fish blood would not take up as much carbon dioxide as normal blood of a species. It is generally recognized that hemoglobin plays an important rôle in the transportation of carbon dioxide. This has been shown by Van Slyke (1921) and many other workers. However, just how close a relationship there is between the hemoglobin concentration and the ability of fish blood to carry carbon dioxide cannot be stated at this time.

The greater concentration of hemoglobin in mackerel blood may also account for the fact that it is easier to demonstrate greater carbon dioxide absorption by its reduced than by its oxygenated blood, than to do it with either toadfish or sea robin blood.

The small amount of carbon dioxide found in the circulating blood of these fishes is in agreement with the findings of Kokubo (1930) for certain other marine species. At the same time the relatively poor buffering ability of their blood agrees with data on other forms presented by Collip (1920), Wastl (1928), and Kokubo (1930). The facts that there is little carbon dioxide normally present in the blood of these fishes, and that it is poorly buffered against carbon dioxide, again suggest an adjustment of the bloods to sea water. There is a low carbon dioxide tension in the gill of a marine fish, a fact necessarily correlated

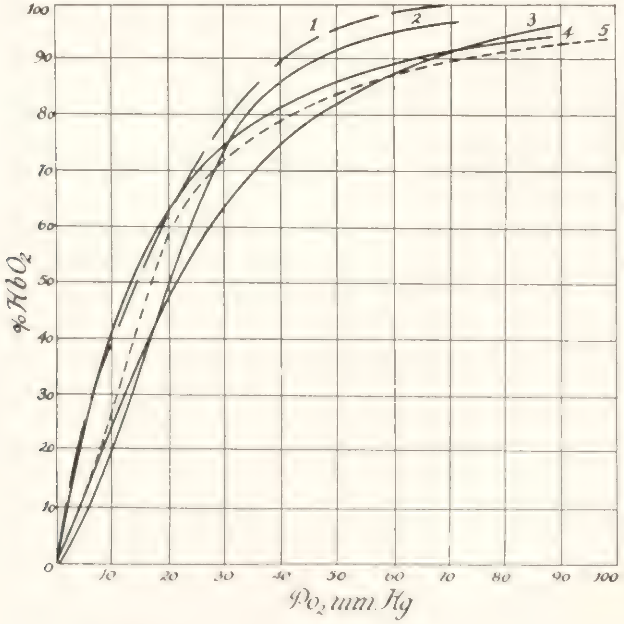

FIG. 13. Comparative oxygen dissociation curves. Curve 1 is for toadfish blood at 20° C. and 1 mm. carbon dioxide; curve 2 for human blood at 37.5° C. and 20 mm. carbon dioxide; curve 3 for turtle blood at 25° C. and 40 mm. carbon dioxide; curve 4 for carp blood at 18° C. and 30 mm. carbon dioxide; and curve 5 for mackerel blood at 20° C. and 1 mm. carbon dioxide tension.

with the low carbon dioxide tension in sea water. At the same time, because of low metabolic rate, a fish produces relatively small quantities of carbon dioxide. Mammalian blood must, by virtue of the high alveolar carbon dioxide tension and the greater metabolic activity on the part of the animal, be prepared to handle larger quantities of carbon dioxide than the blood of a fish. The situation as it stands appears to point to adjustment on the part of both fish and mammal blood to the particular physiological, morphological, and ecological differences that concern the two types of vertebrates.

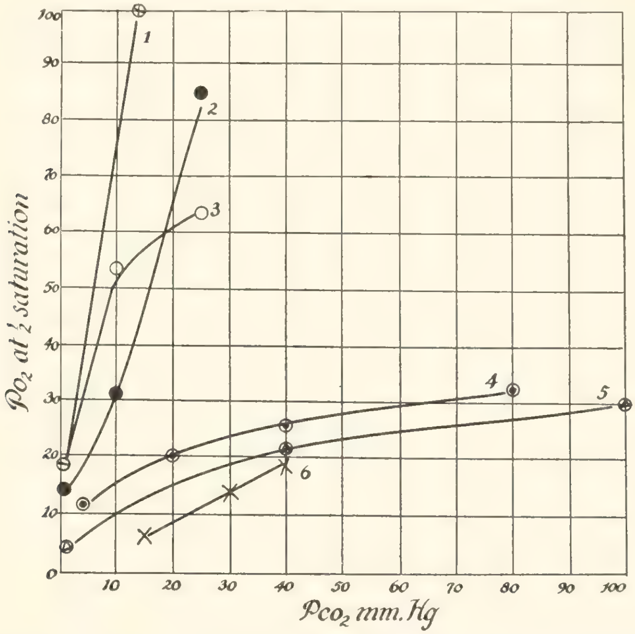

FIG. 14. Effect of carbon dioxide on the "unloading tension" (Po_2 when blood is half saturated) of various vertebrate bloods. Curve 1 is for sea robin blood at 20° C.; curve 2 for toadfish blood at 20° C.; curve 3 for mackerel blood at 20° C.; curve 4 for human blood at 37.5° C.; curve 5 for turtle blood at 25° C.; and curve 6 for carp blood at 18° C.

The calculated pH of fish blood is less than that of sea water. One may wonder how the blood maintains a lower pH. The facts that the blood is poorly buffered, and that it maintains a carbon dioxide tension normally higher than that of sea water probably account for the lower pH.

In comparing the data presented in this paper with similar data on other vertebrates, several interesting differences are brought out. In Fig. 13 a family of oxygen dissociation curves is shown. Conditions have been chosen in such a manner as to make the curves fairly near alike. The oxygen dissociation curve for human blood has been constructed from the data of Bock, Field, and Adair (1924); that for the turtle from Southworth and Redfield's (1926) work; and that for the carp from Wastl's (1928) data. The most noticeable thing about these curves is the diversity of conditions under which they were established. The only way one can make them resemble each other fairly closely is to establish them under widely different conditions of temperature and carbon dioxide tension.

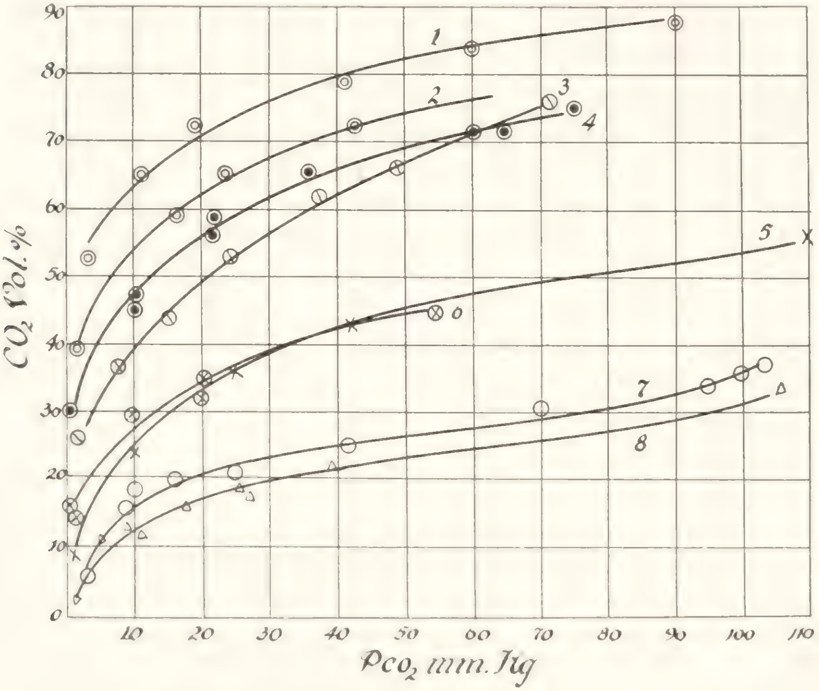

FIG. 15. Comparative carbon dioxide-absorption curves for reduced blood (except for turtle). Curves 1 and 2 are for turtle blood at 25° C.; curve 3 for human blood at 15 °C.; curve 4 for frog blood at 15° C.; curve 5 for mackerel blood at 20° C.; curve 6 for carp blood at 18° C.; curve 7 for sea robin blood at 20° C.; and curve 8 for toadfish blood at 20° C.

In order to show how these same bloods are affected differently by carbon dioxide Fig. 14 has been constructed. One can see at once that the effect of carbon dioxide on marine fish blood is profoundly different from its effect on either human, turtle, or carp blood.

The foregoing comparisons point out well the specificity of hemoglobin in nature that Barcroft (1928) stresses. The significance of specificity is great. Were all hemoglobins alike many animals would not be able to exist under the conditions of their environment, or of their assumed structural and functional characteristics.

For the purpose of showing the differences between the carbon dioxide-absorption curves of various vertebrate bloods Fig. 15 is presented. The data plotted are for reduced blood, except in the case of the turtle. The curves for human, frog, and carp bloods have been constructed from the data of Wastl and Seliškar (1925), and Wastl (1928); and those for the turtle from Southworth and Redfield's (1926) data. The curves show that the blood of fishes is characterized by a relatively weak, those of the frog and turtle by a relatively strong, and that of the human by a more or less intermediate carbon dioxide-combining power. Human blood yields the steepest carbon dioxide-absorption, which means that it is buffered the best. These curves have been plotted at as near the same temperature in all cases as possible, since it has been shown by Warburg (1922), Stadie and Martin (1924), and Cullen, Keeler, and Robinson (1925) that temperature affects the carbon dioxide-combining power of blood.

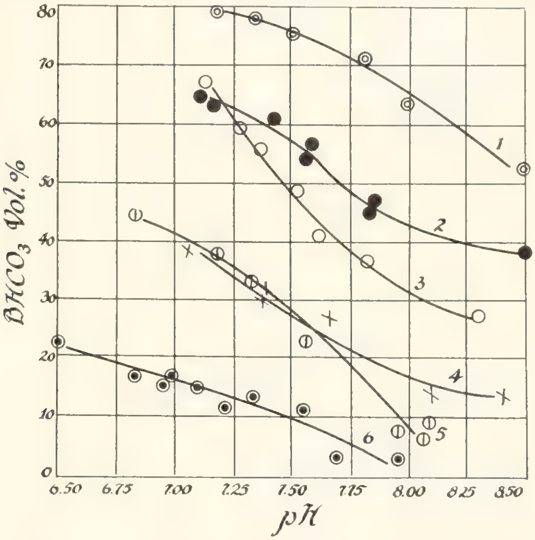

FIG. 16. Comparative BHCO$_3$: pH curves for reduced blood (except for turtle). Curve 1 is for turtle blood at 25° C.; curve 2 for frog blood at 15° C.; curve 3 for human blood at 15° C.; curve 4 for carp blood at 18° C.; curve 5 for mackerel blood at 20° C.; and curve 6 for toadfish blood at 20° C.

In order that the buffering ability of several vertebrate bloods might be compared Figs. 16 and 17 were constructed. Data other than the author's have been taken from the previously mentioned sources and the pH or cH calculated on a basis comparable to the calculations made for marine fish blood. In Fig. 16 the BHCO$_3$: pH relationships are shown; in Fig. 17 the $10^{-8} \times$ cH : P_{CO_2} relationships. In the first figure the more nearly parallel the curve runs with respect to the abscissa the more poorly the blood is buffered. The results here indicate that toadfish blood is the poorest buffered, while human blood is the best buffered. There appears to be little difference in the other bloods. In the second figure the steeper the curve is, the poorer the blood is buffered against carbon dioxide. The results obtained here indicate that toadfish and sea robin blood are relatively poorly buffered, while frog, turtle, and human blood are relatively well buffered. Carp and mackerel blood are more or less intermediate with respect to the others, resembling, however, the bloods of the higher vertebrates slightly more than those of the toadfish and sea robin.

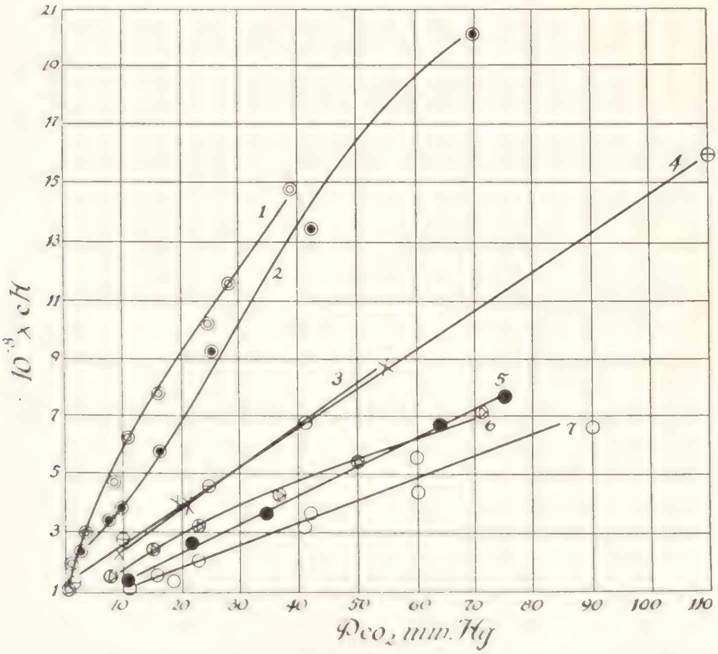

FIG. 17. Comparative $10^{-8} \times$ cH : P_{CO_2} curves for reduced blood (except for turtle). Curve 1 is for toadfish blood at 20° C.; curve 2 for sea robin blood at 20° C.; curve 3 for carp blood at 18° C.; curve 4 for mackerel blood at 20° C.; curve 5 for frog blood at 15° C.; curve 6 for human blood at 15° C.; and curve 7 for turtle blood at 25° C.

There is another point of interest about Figs. 16 and 17. Regardless of the slope of the curves, at any given pH the bloods do not have the same $BHCO_3$ content; likewise at any given cH they are not subjected to the same carbon dioxide tension. This may be explained by the fact that the carbon dioxide-absorption level is quite different for the different bloods. The higher the level at a given carbon dioxide tension the more the hydrogen ion concentration is displaced in the alkaline direction. Southworth and Redfield (1926) have shown that as far as turtle blood is concerned the characteristically high level of the carbon dioxide-absorption curve is due to high $BHCO_3$ in the plasma and the relatively small amount of hemoglobin present to act as an acid in dissociating carbon dioxide from its salt. Perhaps the same thing holds true for frog blood. It is interesting to note that in the case of toadfish and sea robin blood the dissociation of carbon dioxide is quite complete even though there is a low hemoglobin concentration.

The differential buffering ability of the bloods may possibly be explained on the basis of the nature of the adjustments that vertebrates have undergone in going from an aquatic to a terrestial environment. The acquirement of lungs and a higher rate of metabolism has made necessary a greater buffering defense.

Summary

1. The oxygen capacities of marine fish bloods are quite different for different species. The greatest difference is between the typically sluggish and active forms, the former having bloods of low, and the latter bloods of high oxygen capacity. There is a general correlation between oxygen capacity and corpuscle count, corpuscle volume, and iron content.

2. Studies on the oxygen dissociation curves of marine fish hemoglobin, and on the effect of carbon dioxide on the oxygen capacity have brought forth the suggestion that the effect of carbon dioxide on the hemoglobins of these fishes is not solely on their oxygen dissociation constants, but that there is an inactivation of certain of the prosthetic groups concerned in binding oxygen in the hemoglobin molecule, causing a marked decrease in oxygen-combining power of the bloods. The most marked evidence of inactivation occurs at definite ranges of carbon dioxide tension and pH for the different bloods.

3. The carbon dioxide-combining power of fish bloods appears to be correlated with hemoglobin concentration. Mackerel blood with high hemoglobin absorbs more carbon dioxide than toadfish blood, which has a low hemoglobin concentration.

4. Reduced fish blood will absorb slightly more carbon dioxide than oxygenated blood. For sea robin and toadfish bloods the range of carbon dioxide tension where this can be demonstrated is short, being between about 2 and 25 mm., while it is longer for mackerel blood, being about 2 to 95 mm.

5. There is a differential buffering ability shown by these bloods, mackerel blood being buffered the best and toadfish the poorest.

6. Comparative studies of vertebrate bloods strengthen the idea of specificity of hemoglobins. Those of the marine fishes are far more sensitive to carbon dioxide than those of the carp, turtle, and human.

7. Comparative studies on carbon dioxide transportation show that turtle and frog bloods have a relatively great, fishes a relatively small, and human blood a more or less intermediate carbon dioxide-combining power. The bloods also vary considerably in their buffering capacity, human blood having the greatest and toadfish blood the least.

8. The general results of this investigation point to an adjustment on the part of the blood of marine fishes to a sea-water environment, and the habits or characteristics of the fishes. At the same time the comparative studies indicate marked differences between the bloods of fishes and terrestrial vertebrates. These differences can perhaps be accounted for on the basis of the new morphological and physiological features that terrestrial vertebrates have acquired, along with change in environment, which have made necessary correlative changes in the respiratory function of the blood.

I wish to express to Dr. F. G. Hall my profound appreciation for the many timely suggestions and criticisms that he offered during the progress of this work. I wish to thank various members of the Duke University Zoölogy Department, and of the United States Bureau of Fisheries, particularly Dr. I. E. Gray, Dr. O. E. Sette, Dr. A. S. Pearse, and Mr. S. R. Tipton. I also wish to thank Dr. A. C. Redfield of Harvard University for the many helpful suggestions that he has given me.

LITERATURE CITED

Adair, G. S., 1925. The Hemoglobin System. VI. The oxygen dissociation curve of hemoglobin. *Jour. Biol. Chem.*, **63**: 539.

Austin, J. H., G. E. Cullen, A. B. Hastings, F. C. McLean, J. P. Peters, and D. D. Van Slyke, 1922. Studies of Gas and Electrolyte Equilibria in Blood. I. Technique for collection and analysis of blood, and for its saturation with gas mixtures of known composition. *Jour. Biol. Chem.*, **54**: 121.

Barcroft, J., 1928. The Respiratory Function of the Blood. Part II. Hemoglobin. Cambridge.

Bock, A. V., H. Field, and G. S. Adair, 1924. The Oxygen and Carbon Dioxide Absorption Curves of Human Blood. *Jour. Biol. Chem.*, **59**: 353.

Bohr, C., 1905. Absorptions coëfficienten des Blutes und des Blutplasmas für Gase. *Skand. Arch. f. Physiol.*, **17**: 104.

Christiansen, J., C. G. Douglas, and J. S. Haldane, 1914. The Absorption and Dissociation of Carbon Dioxide by Human Blood. *Jour. Physiol.*, **48**: 244.

Collip, J. B., 1920. The Alkali Reserve of Marine Fish and Invertebrates. The excretion of carbon dioxide. *Jour. Biol. Chem.*, **44**: 329.

Cullen, G. E., H. R. Keller, and H. W. Robinson, 1925. The pK′ of the Henderson-Hasselbalch Equation for Hydrion Concentration of Serum. *Jour. Biol. Chem.*, **66**: 301.

GAARDER, T., 1918. Über den Einfluss des Sauerstoffdruckes auf den Stoffwechsel. II. Nach Versuchen an Karpfen. *Biochem. Zeitschr.*, **89**: 94.

GRAY, I. E., AND F. G. HALL, 1930. Blood Sugar and Activity in Fishes with Notes on the Action of Insulin. *Biol. Bull.*, **58**: 217.

HALL, F. G., I. E. GRAY, AND S. LEPKOVSKY, 1926. The Influence of Asphyxiation on the Blood Constituents of Marine Fishes. *Jour. Biol. Chem.*, **67**: 549.

HALL, F. G., AND I. E. GRAY, 1929. The Hemoglobin Concentration of the Blood of Marine Fishes. *Jour. Biol. Chem.*, **81**: 589.

HALL, F. G., 1928. Blood Concentration in Marine Fishes. *Jour. Biol. Chem.*, **76**: 623.

HALL, F. G., 1929. The Influence of Varying Oxygen Tensions upon the Rate of Oxygen Consumption in Marine Fishes. *Am. Jour. Physiol.*, **88**: 212.

HALL, F. G., 1930. The Ability of the Common Mackerel and Certain Other Marine Fishes to Remove Dissolved Oxygen from Sea Water. *Am. Jour. Physiol.*, **93**: 417.

HASSELBALCH, K. A., 1917. Die Berechnung der Wasserstoffzahl des Blutes aus der freien und gebundenen Kohlensäure desselben, und die Sauerstoffbindung des Blutes als Funktion der Wasserstoffzahl. *Biochem. Zeitschr.*, **78**: 112.

HASTINGS, A. B., AND J. SENDROY, JR., 1925. The Effect of Variation in Ionic Strength on the Apparent First and Second Dissociation Constants of Carbonic Acid. *Jour. Biol. Chem.*, **65**: 445.

HASTINGS, A. B., J. SENDROY, JR., AND D. D. VAN SLYKE, 1928. Studies of Gas and Electrolyte Equilibria in Blood. XII. The value of pK′ in the Henderson-Hasselbalch equation for blood serum. *Jour. Biol. Chem.*, **79**: 183.

HENDERSON, L. J., 1908. The Theory of Neutrality Regulation in the Animal Organism. *Am. Jour. Physiol.*, **21**: 427.

HENDERSON, T., 1918. Applications of Gas Analysis. IV. The Haldane gas analyzer. *Jour. Biol. Chem.*, **33**: 31.

JOBES, F. W., AND M. E. JEWELL, 1927. Studies on the Alkali Reserve of the Blood of Ameriurus nebulosus from Acid and Basic Waters. *Trans. Am. Micros. Soc.*, **46**: 175.

JOLYET, F., AND P. REGNARD, 1877. Recherches physiologiques sur la respiration des animaux aquatiques. 1 and 11. *Arch. Physiol. Norm. et Path.*, **4**: 44, 584.

KAWAMOTO, N., 1929. Physiological Studies on the Eel. I. The seasonal variation of the blood constituents. II. The influence of temperature and of the relative volume of the red corpuscles and plasma upon the haemoglobin dissociation curve. *Sci. Rep. Tohoku Imper. Univ.*, Series 4, **4**: 635.

KOKUBO, S., 1927. Contribution to the Research on the Respiration of Fishes. I. On the hydrogen ion concentration and the CO_2-gas content and capacity of fish blood. *Sci. Rep. Tohoku Imper. Univ.*, Series 4, **2**: 325.

KOKUBO, S., 1930. Contribution to the Research on the Respiration of Fishes. II. Studies on the acidosis of fishes. *Sci. Rep. Tohoku Imper. Univ.*, Series 4, **5**: 249.

KROGH, A., AND I. LEITCH, 1919. The Respiratory Function of the Blood in Fishes. *Jour. Physiol.*, **52**: 288.

NICLOUX, M., 1923. Action de l'oxyde de carbone sur les poissons et capacité respiratoire du sang de ces animaux. *Compt. r. Soc. Biol.*, **89**: 1328.

PETERS, J. P., H. A. BULGER, AND A. J. EISENMAN, 1923. Studies of the Carbon Dioxide Absorption Curve of Human Blood. I. The apparent variations of pK_1 in the Henderson-Hasselbalch equation. *Jour. Biol. Chem.*, **55**: 687.

PETERS, J. P., H. A. BULGER, AND A. J. EISENMAN, 1924. Studies of the Carbon Dioxide Absorption Curve of Human Blood. IV. The relation of the haemoglobin content of the blood to the form of the carbon dioxide-absorption curve. *Jour. Biol. Chem.*, **58**: 747.

POWERS, E. B., 1922. The Alkaline Reserve of the Blood of Fish in Relation to the Environment. *Am. Jour. Physiol.*, **61**: 380.

Redfield, A. C., T. Coolidge, and A. L. Hurd, 1926. The Transport of Oxygen and Carbon Dioxide By Some Bloods Containing Hemocyanin. *Jour. Biol. Chem.*, **69**: 475.

Redfield, A. C., and E. D. Mason, 1928. The Combination of Oxygen and Hydrochloric Acid with the Hemocyanin of Limulus. *Am. Jour. Physiol.*, **85**: 401.

Southworth, F. C., and A. C. Redfield, 1926. The Transport of Gas by the Blood of the Turtle. *Jour. Gen. Physiol.*, **9**: 387.

Stadie, W. C., and K. A. Martin, 1924. The Thermodynamic Relations of the Oxygen- and Base-combining Properties of Blood. *Jour. Biol. Chem.*, **60**: 191.

Stadie, W. C., and E. R. Hawes, 1928. Studies on the Oxygen-, Acid-, and Base-combining Properties of Blood. IV. The apparent first dissociation constant, pK_1', of carbonic acid and the activity coefficient of the bicarbonate ion in solutions of hemoglobin, methemoglobin, cyanhemoglobin, and nitric oxide hemoglobin at varying ionic strengths. *Jour. Biol. Chem.*, **77**: 265.

Stadie, W. C., 1928. Studies on the Oxygen-, Acid-, and Base-combining Properties of Blood. V. Extension of the Debye-Hückel theory of ionic interaction to hemoglobin, bicarbonate-sodium chloride systems. *Jour. Biol. Chem.*, **77**: 303.

Trendelenburg, P., 1912. Über die Sauerstofftension im Blute von Seefischen. *Zeitschr. f. Biol.*, **57**: 495.

Van Slyke, D. D., 1921. The Carbon Dioxide Carriers of the Blood. *Physiol. Rev.*, **1**: 141.

Van Slyke, D. D., H. Wu, and F. C. McLean, 1923. Studies of Gas and Electrolyte Equilibria in the Blood. V. Factors controlling the electrolyte and water distribution in the blood. *Jour. Biol. Chem.*, **56**: 765.

Van Slyke, D. D., and J. M. Neill, 1924. The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement. I. *Jour. Biol. Chem.*, **61**: 523.

Van Slyke, D. D., A. B. Hastings, C. D. Murray, and J. Sendroy, Jr., 1925. Studies of Gas and Electrolyte Equilibria in Blood. VIII. The distribution of hydrogen, chloride, and bicarbonate ions in oxygenated and reduced blood. *Jour. Biol. Chem.*, **65**: 701.

Van Slyke, D. D., and J. Sendroy, Jr., 1927. Carbon Dioxide Factors for the Manometric Blood Gas Apparatus. *Jour. Biol. Chem.*, **73**: 127.

Warburg, E. J., 1922. Studies on Carbonic Acid Compounds and Hydrogen Ion Activities in Blood and Salt Solutions. A contribution to the theory of the equation of L. J. Henderson and K. A. Hasselbalch. *Biochem. Jour.*, **16**: 153.

Wastl, H., and A. Seliškar, 1925. Observations on the Combination of Carbon Dioxide in the Blood of the Bull Frog (*Rana catesbiana*). *Jour. Physiol.*, **60**: 264.

Wastl, H., 1928. Beobachtungen über die Blutgase des Karpfenblutes. *Biochem. Zeitschr.*, **197**: 363.

THE RESPIRATION OF PUFFER FISH

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The mechanism of respiration of fishes which live in the sea offers an attractive and productive subject for study. The ocean is stable and uniform and therefore a favorable environment for living organisms. An abundant supply of oxygen is usually present. The hydrogen ion concentration varies only in a range which is close to the optimum for physiological processes, especially for the elimination of carbon dioxide. The temperature of the ocean as compared with freshwater and land conditions is relatively uniform. Moreover, sea water is similar in constitution to the internal fluids of marine organisms. Since most vertebrates have mechanisms for maintaining conditions within their bodies more or less constant and since fishes are the last of typically marine vertebrates to evolve, it seems important to study the factors which vary in sea water and which in some manner influence the respiratory exchange of gases between fishes and their surroundings.

Fishes breathe dissolved gases from water which they pump over their gills. The mechanism for external respiration consists in most fishes of rhythmical suction of water into the oral cavity and its subsequent expulsion through the gill clefts. During inspiration the mouth is opened and the oral cavity enlarged by the lateral expansion of its walls. When the oral cavity is closed the expiratory process begins. By the lateral contraction of the oral walls water is driven through the gill clefts and over the gill filaments. The branchial arches are spread apart during the expiratory phase, thus permitting all of the filaments to come into direct contact with the circulating water. The gas exchange between the blood and water takes place through the walls of the filaments.

Considering the general mechanics of external respiration as shown by fishes, several problems come to mind. How much water is pumped in a single respiratory cycle? How much of the dissolved oxygen is removed from sea water as it passes the gills? When an increased oxygen supply is required, which plays the more important rôle—an increase in the ability of the gills to absorb oxygen from the sea water; an increase in the volume of water pumped by a single respiratory cycle; or an increase in the number of respiratory cycles per unit time? Consideration is given to each of these possibilities in the following pages.

Three physico-chemical factors which may vary in the external medium and affect the equilibrium which the organism maintains in its internal environment are temperature, oxygen tension, hydrogen ion concentration (carbon dioxide tension and hydrogen ion concentration *per se*). By varying these factors in the investigations to be described, a means of studying certain phases of the general problem of respiration was found.

The most extensive studies bearing on the problems of fish respiration are those of Winterstein (1908). He used the fresh-water fish, *Leuciscus erythrophtalmus.* Fishes under observation were held fast by a clamp, while a constant stream of water of a known oxygen tension was passed over the gills by means of a thick canula fastened in the mouth of the fish. The amount of oxygen used up was determined. This is perhaps the simplest and most direct method that has been devised for the determination of the respiratory exchange in fishes. However, as Winterstein has pointed out, one must keep in mind that the fishes are breathing somewhat abnormally. When fishes have water forced over their gills, they may not respire in the same way as if they were pumping the water over the gills in the natural manner. He concludes from his experiments that the oxygen consumption is independent of oxygen tension of the surroundings within wide limits of magnitude, and that the utilization of oxygen is in inverse proportion to the flowing velocity. Henze (1910) has also shown that oxygen consumption in certain fishes is not influenced to any great extent by the oxygen tension of the surrounding water. His results are expressed in arbitrary values and are not particularly constant.

Gaarder (1918) has performed an interesting experiment on the fresh water carp. His paper is stimulating and thoughtful. However, it discusses only a few analyses and has the disadvantage that the gills were subjected to forced ventilation and therefore were perhaps not functioning naturally. Gaarder had the misfortune, it appears, of being quoted inaccurately, being said to conclude that oxygen consumption is within wide limits proportional to oxygen tension. Another author quotes him as believing that oxygen consumption is independent of oxygen tension. The writer understands Gaarder's conclusion to be that consumption is uninfluenced so long as the hemoglobin of the blood is not fully saturated; when oxygen and the oxygen tension of the physically dissolved oxygen is raised considerably, then oxygen consumption shows an increase.

Powers (1922, 1929) and Powers and Shipe (1928) have shown that carbon dioxide tension and pH have a pronounced effect on the respiration of fishes. Powers (1930) has given an excellent summary of the relation between pH and aquatic animals.

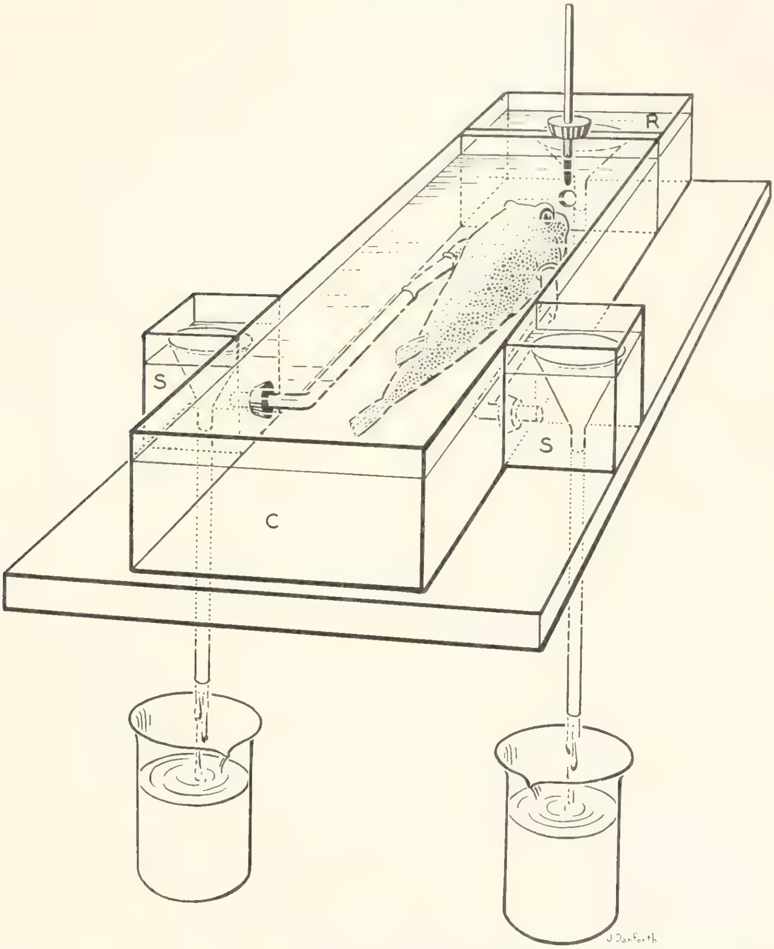

Fig. 1. Apparatus used for the determination of the influence of environmental factors on the respiration of puffer fishes.

Methods

The puffer fish, *Spheroides maculatus* (Bloch and Schneider), was used in the writer's investigations because it was particularly adapted to such a study. The rounded shape of the opercular aperture, which is considerably reduced in size as compared with other fishes, makes this species especially advantageous. Glass tubes may be inserted through the opercular openings without apparent injury. All of the water pumped by the fish for respiratory purpose will then flow through the glass tubes and samples can be collected for analyses. Fishes carrying such tubes will lie quietly for hours apparently breathing normally, and will live in this condition for several weeks. Tubes were inserted in the opercular openings of puffers about three or four days previous to using them for the respiration experiments. Thus the animals became accustomed to breathing in such a manner.

The apparatus used in these experiments is shown in Fig. 1. The fish was submerged in a chamber (*c*), which had a capacity of 4 liters. A reservoir (*R*) to which flowing water was admitted and which also contained a funnel out of which all of the excess water flowed was connected to the chamber by a hole one inch in diameter. Two side compartments (*s*) were so arranged that they could be connected with the glass tubes inserted in the opercular opening of the fish. A funnel was placed in each side compartment at the same level as that in the reservoir (*R*). The height of the funnels in each case was adjusted so that before the fish was connected to the side compartments water entering the reservoir would flow out through the funnel in the reservoir but would not flow out through the funnels in the side compartments. Thus only a very slight exertion on the part of the fish was required to pump water from the chamber in which it was submerged to the funnels in the side compartments. Fishes were placed in the chamber so that their mouths were close to the hole leading from the reservoir. Thus a fresh flowing supply of water was always available. It was not found necessary to either anæsthetize these fishes or to clamp them. If they were left undisturbed by outside factors they would remain quiet for hours.

The quantity of water pumped per minute was measured by use of volumetric flasks placed under the funnels, and a stopwatch. The quantity of water pumped through the right and left gill chambers was taken separately. Analyses of the dissolved oxygen was made on the water before it entered the fish's mouth and after it had been pumped into the side compartments. The well known Winkler method as modified by Birge and Juday was employed. Care was taken not to expose the water to air in taking the samples.

In experiments where the influence of temperature was studied, water was cooled to the desired temperature by passing through coils in a constant temperature bath. A range of 10° C. was used since puffers do not readily adjust themselves to a lower temperature than 10°–11° C. or higher than 23°–24° C. The temperature range chosen for this experiment was 12°–22° C.

The hydrogen ion concentration of the water was measured by colorimetric means. Consequently the analyses do not represent a precise measurement or an absolute value since salt errors are introduced. No corrections have been made for salt errors. The pH determinations must be taken only as of relative values. In one type of experiment the hydrogen ion concentration of the water was controlled by the addition of carbon dioxide gas. In a second type hydrochloric acid was added to the water and the carbon dioxide formed was driven off by aëration.

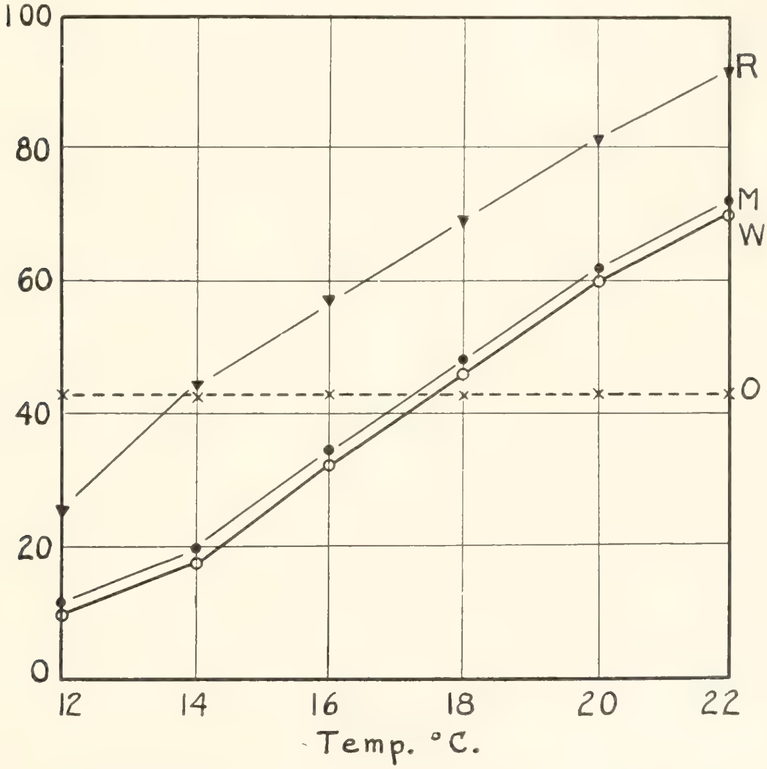

FIG. 2. Graph showing the influence of temperature on the respiration of puffer fishes. Respiratory rhythm (*R*) in respirations per minute; oxygen consumption (*M*) in cc. of oxygen per kilogram per hour; water pumped through branchial cavity (*W*) in deciliters per hour; percentage of dissolved oxygen (*O*) removed from the affluent water.

Sea water of different oxygen tensions was procured by boiling and subsequent mixing with normal sea water. In this manner sea water of any desired oxygen tension could be obtained.

RESULTS

The results of the first experiment are graphically indicated in Fig. 2. They show the influence of temperature on the respiration of puffer fishes. Ten individuals were submitted to various temperatures as indicated on the graph and the results for each were averaged. It may be observed that the respiratory rhythm (*R*), rate of metabolism (*M*), and quantity of water pumped per minute (*W*) increased progressively with increase in the temperature of the surrounding water.

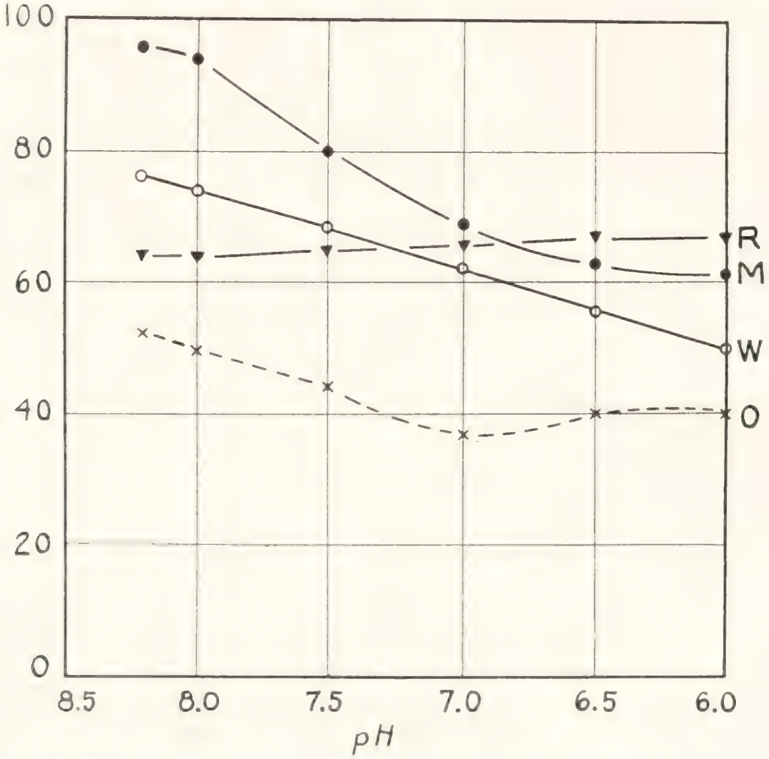

Fig. 3. Graph showing the influence of pH with low CO_2 tensions on respiration of puffer fishes. Scale and legend as in Fig. 2.

The percentage of the dissolved oxygen absorbed from the surrounding water, however, did not increase appreciably. At 20° C., which was approximately the temperature of sea water in the Woods Hole Region at the time these experiments were conducted, puffer fishes had an average rhythm of 80 respirations per minute, pumped 6 liters of water over their gills in an hour, absorbed 45 per cent of the dissolved oxygen from the water, and consumed on the average 62 cc. of oxygen per kilogram of body weight in an hour.

The second experiment shows the effects on the respiration of puffer fishes of varying the hydrogen ion concentration of the surrounding water by the addition of hydrochloric acid to sea water (and subsequent aëration in order to remove excess carbon dioxide). The results obtained with six individuals are averaged and summarized graphically in Fig. 3. They show that decreasing pH *per se* apparently inhibits the rate of metabolism (*M*), and the amount of water pumped by fishes.

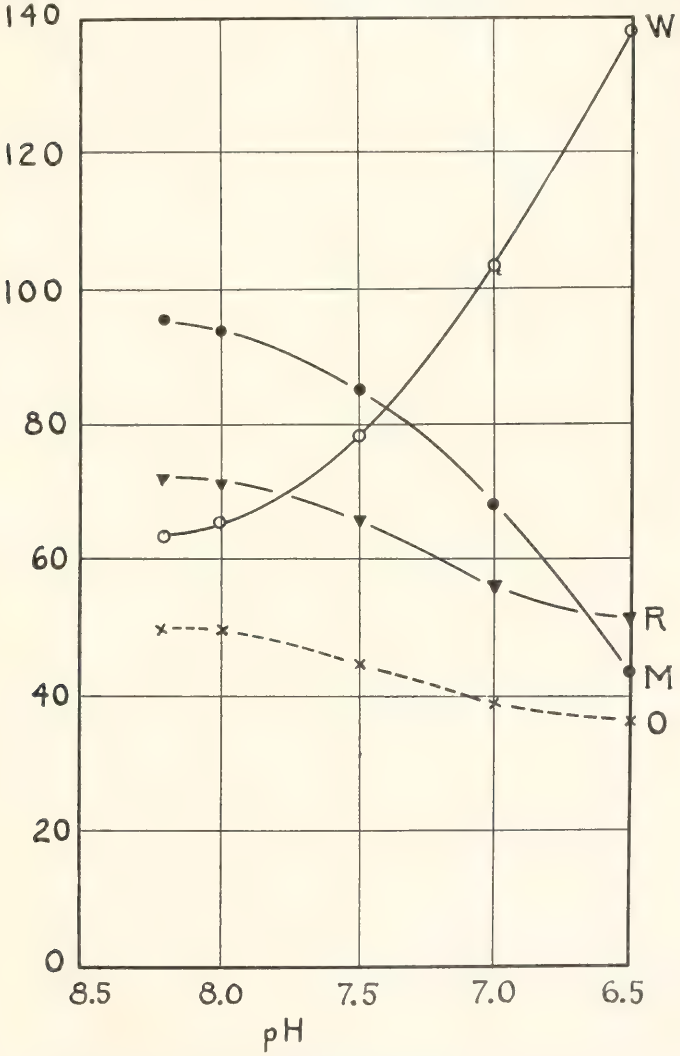

Fig. 4. Graph showing the influence of pH with high CO_2 tensions on respiration of puffer fishes. Scale and legend as in Fig. 2.

The respiratory rhythm (R) is affected slightly. The percentage of oxygen absorbed (O) decreases with increasing acidity.

The third experiment was devised to show how dissolving carbon dioxide would affect respiration as compared with the effect of pH produced by hydrochloric acid in the previous experiment. Figure 4 represents the average results obtained with puffer fishes. These indicate that variations in carbon dioxide concentration expressed in terms of the pH of the water which contains it have a much greater influence on respiration of fishes than variations in pH due to other factors. The quantity of water pumped (W) was markedly accelerated when the sea water approached the acid side of neutrality. The rate of metabolism was greatly inhibited. The respiratory rhythm decreased in rate accordingly. Fishes died when the pH was lowered below 6.5, while in the previous experiment no difficulty was experienced in submitting individuals to a pH of 6.0.

TABLE I

The percentage of dissolved oxygen absorbed by puffer fish from sea water of varying oxygen tensions at 20° C.

Dissolved Oxygen in cc. Per Liter		Percentage of Dissolved Oxygen Absorbed
Affluent Water	Effluent Water	
4.68	2.16	46
4.00	1.84	46
3.10	1.49	48
2.31	1.10	47
1.14	0.58	45
0.98	0.45	46

The purpose of the fourth experiment was to determine the percentage of oxygen which fishes absorbed at different oxygen tensions. These determinations were made on eight individuals at a constant temperature of 20° C. Dissolved oxygen analyses were made on the affluent water which was being sucked into the mouth of the fishes and on the effluent water which was flowing out of the opercular opening after it had passed the gills. The results obtained with each individual were averaged and are shown in Table I. They indicate that fishes are able to absorb from 45 to 48 per cent of the dissolved oxygen from sea water regardless of wide variation in the tension of the dissolved oxygen in the affluent water.

Discussion

It is evident from the foregoing experiments that the puffer fish is able to pump considerable water over the gills. The quantity of water circulated through the gill clefts and over the gill filaments varies under different circumstances (Fig. 2). When the temperature of the inspired water is increased, the oxygen consumption increases progressively. Concomittantly more water is pumped by the fish. However, the quantity of water which is pumped by a single inspiration and expiration varies but little and remains relatively constant through a wide range of temperature changes. The rate of respirations per minute, on the other hand, shows a parallel increase with that of water pumped and oxygen consumed by the organism. Similarly, the quantity of dissolved oxygen removed does not seem related or influenced by the oxygen consumed, but remains at a fairly constant level. Between temperatures of 12° and 22° C. the variation in the percentage of oxygen removed from the inspired water was between 44 and 45 per cent. It seems, therefore, that the need for an increased quantity of oxygen with increasing temperature is obtained mainly by regulation of the respiratory rhythm and not by the quantity of water pumped on each inspiration or the quantity of oxygen removed from the inspired water.

The gills are apparently a very efficient mechanism through which oxygen is absorbed into the blood. Gill filaments are made of numerous lamellæ, thereby increasing the absorptive surface. Capillaries supply the lamellæ with blood which passes into the general circulation. The outer membrane of the gill filaments is very thin, only a few microns in thickness. Through this membrane dissolved molecular oxygen passes from the sea water into the blood and is there bound by hemoglobin. A small quantity of molecular oxygen will also be found in the blood in the same state as in sea water, *i.e.,* physically dissolved. The oxygen capacity of the blood of puffer fishes has been found to range from 8 to 10 volumes per cent.

When water is pumped into the mouth of the puffer, it is forced out between the branchial arches in such a way that a great proportion of it comes into contact with the gill lamellæ. The gills are flattened and elongated and are fairly close together when water is forced past them. Thus their anatomical arrangement is particularly advantageous. Several factors are to be considered in properly interpreting their function. When a stream of water passes through a branchial cleft its velocity will be greatest in the middle of the stream and least nearest the lamellæ. Relatively more oxygen will consequently be absorbed from the water nearer the lamellæ than from that further away. If oxygen is to be absorbed from the water moving at the higher velocity it must diffuse rather rapidly. The rate of diffusion will depend upon the pressure gradient.

Thus the efficiency of the respiratory mechanism may in a way be determined by comparison of the gas tensions of the affluent and effluent water. Figure 1 shows that at 20° C. puffers pump an average of 6 liters of water per hour over their gills, and that 45 per cent of the dissolved oxygen was removed from affluent water. This indicates a very effective aëration of the gills. Such a conclusion is further substantiated by the fourth experiment, in which the oxygen tension of the affluent water was changed through a series of tensions ranging from 0.9 cc. per liter to 4.8 cc. per liter. It was found that about the same percentage of oxygen was removed regardless of the oxygen tension. The percentage varied only from 45 to 48 per cent. This indicates that the respiratory mechanism of gill aëration is equally efficient over quite a wide range of oxygen tensions.

An interesting point which must be considered in investigations concerned with the respiration of fishes is the absence of any mechanical buffering means such as is present in the alveolar air of air-breathing animals. Mammals particularly have a residual air supply which maintains a fairly constant CO_2 and O_2 tension so that moderate irregularities in breathing only slightly change the gas tensions of the alveolar air. Fishes, however, have their gills directly exposed to water and have nothing comparable to alveolar air tensions. Their gills are directly exposed to the gas tension of the water in which they live. They have apparently no means by which the gas tensions to which their gills are subjected may be altered. Since the amount of CO_2 in sea water is low, the CO_2 tension of the water surrounding the gill filaments would be much lower than the CO_2 tension in the alveolar air of lung-breathing vertebrates. Investigations are now being conducted to determine the CO_2 tension of fishes blood and its rôle in the respiratory function of the blood.

SUMMARY

1. A method is described for studying environmental factors which affect the respiration of fishes.

2. An increase in temperature of water surrounding puffer fishes is followed by increased oxygen consumption by the fishes, a greater quantity of water pumped through the branchial chamber, and a faster respiratory rhythm. The percentage of dissolved oxygen absorbed remains constant at all temperatures observed.

3. Increase in hydrogen ion concentration inhibits oxygen consump-

tion by marine fishes, but addition of CO_2 has a more pronounced effect than addition of HCl at the same pH.

4. The results indicate that marine fishes apparently remove dissolved oxygen from sea water by an efficient mechanism of gill aëration. Fishes absorbed about 46 per cent of dissolved oxygen from sea water at all observed oxygen tensions.

BIBLIOGRAPHY

GAARDER, T., 1918. *Biochem. Zeitschr.*, **89**: 94.
HENZE, M., 1910. *Biochem. Zeitschr.*, **26**: 255.
POWERS, E. B., 1922. *Jour. Gen. Physiol.*, **4**: 305.
POWERS, E. B., 1929. *Ecology*, **10**: 97.
POWERS, E. B., 1930. *Am. Nat.*, **64**: 342.
POWERS, E. B., AND L. M. SHIPE, 1928. *Pub. Puget Sound Biol. Sta.*, **5**: 365.
WINTERSTEIN, H., 1908. *Pflüger's Arch.*, **125**: 73.

THE RATE OF OXYGEN CONSUMPTION OF ASTERIAS EGGS BEFORE AND AFTER FERTILIZATION[1]

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I

Since the account of Loeb and Wasteneys (1912) nineteen years ago, no data have been made available on the rate of oxygen consumption of *Asterias* eggs before and after fertilization. In view of the importance of such studies for the understanding of the mechanism of development as well as that of cellular oxidation, it was considered desirable to reinvestigate the subject, using the microrespirometer technic. This method has the advantage over the Winkler method, which Loeb and Wasteneys used, in that slight changes in rate of oxygen consumption can be detected at rather short consecutive time intervals.

II

The microrespirometers employed were those described by Warburg (1926). Conical vessels of about three cubic centimeters capacity with side arms and cylindrical insets for alkali were used. Half a cubic centimeter of egg suspension was placed in each vessel. In the experiments with fertilized eggs, 0.1 cc. of sperm suspension was introduced either directly into the chamber containing the eggs or into the side-arm to be mixed with the eggs after a number of readings on the unfertilized eggs had been taken. The experiments were conducted at 23.0° C. and the manometers shaken at the rate of 70 complete oscillations a minute with an amplitude of 15 cm., which was demonstrated to be adequate to insure the requisite mixing.

Eggs from single animals were used. The gonads were removed from the animals with a pair of forceps after partially detaching the appendages, and placed in about twenty-five cubic centimeters of sea water. After the eggs were shed, they were filtered through cheese cloth into a 100 cc. beaker filled with sea water and concentrated by decanting the supernatant liquid. A portion of the eggs was examined about twenty minutes after removal for maturation, and only those lots of eggs with 50 per cent or more maturation were used in the experiments. At the end of an experiment, the eggs were examined again and the percentages of maturation or cleavage were recorded. The experiments were conducted during July and August at a time past the height of the breeding season and the number of satisfactory experiments available for analysis was relatively few. However, all experiments showed good agreement qualitatively, and only the typical ones are given here.

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[1] Supported in part by a grant from the Rockefeller Foundation to the University of Chicago.

III

A series of experiments was conducted in the following manner: Half-cc. portions of egg suspension were placed in four vessels with the sperm in the side arms. After a number of readings at 5-minute intervals with the eggs unfertilized, the sperm in the side arms of three of the vessels was mixed with the eggs, an operation requiring less than a minute, and readings at 5-minute intervals were continued for 100 minutes. The data are plotted in Fig. 1. In these graphs the ordinate represents the relative rates of oxygen consumption and the abscissæ, time in minutes. Lines *B*, *C* and *D* are obtained from experiments in which the eggs were fertilized after the fourth reading: line *A* represents the control in which the eggs and the sperm remained separate during the experiment. The arrow points to the time of fertilization. The results indicate that there is no change, either temporary or permanent, in the rate of oxygen consumption during the first 100 minutes after fertilization, and the scattering of the points is almost identical in the fertilized and the unfertilized eggs. This scattering is due, presumably, to errors in reading the small changes on the manometers. The result confirms the findings of Loeb and Wasteneys, and is unlike the case of *Arbacia* eggs (*e.g.*, Tang, 1931). It may be remarked that the per-

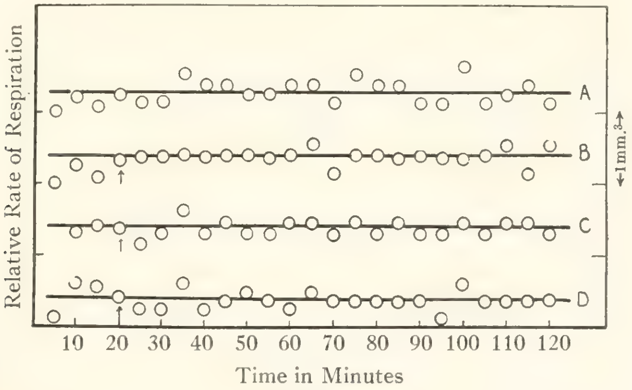

FIG. 1.

centages of fertilization and cleavage were somewhat low (less than 50 per cent of all eggs), although samples from the same lot of eggs kept in a Syracuse watch glass at room temperature (25° C.) showed as much as 85 per cent cleavage. These low percentages would diminish but not mask the respiratory changes due to fertilization if they were present.

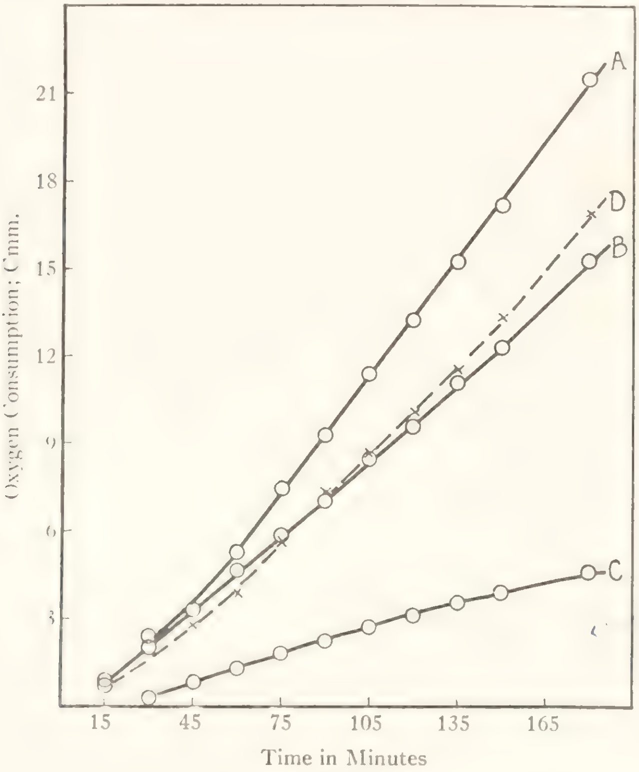

FIG. 2.

In a second series of experiments the eggs in two of the respirometer vessels were fertilized immediately before the experiments were started, two other vessels contained the same amount of eggs but unfertilized, and a fifth vessel contained a known amount of the sperm suspension used in the first two vessels. The rates of oxygen consumption were followed for three hours. When the experiments were performed in this way, over 50 per cent of the eggs had cleaved to 8 and 16-cell stages at the end of the experiment, and over 80 per cent of the eggs had matured. The data are presented in Fig. 2, in which the ordinate represents the amount of oxygen consumption in cubic millimeters and the abscissa, the time in minutes after the closing of the manometers. The values for the fertilized eggs with sperm are plotted as line *A*, those for the unfertilized eggs, *B*; and those for the sperm, *C*. The broken line *D* is the corrected curve for the fertilized eggs minus the sperm, *i.e.*, *A–C*, which falls closely on the curve for the unfertilized eggs.

In some of the experiments, for reasons yet obscure, over 80 per cent of the unfertilized eggs remained immature after three hours in the respirometers although controls in Syracuse watch glasses gave a high percentage of maturation. Like the mature eggs, their rate of oxygen consumption is constant, and for the first hour in the respirometers it is equal to those of the mature and fertilized eggs, becoming slightly lower after the second hour.

The absolute rates of oxygen consumption (Qo_2) for these eggs of the second series during the first hour in the respirometers expressed in terms of cubic millimeters per hour per million eggs (the number being obtained by hemocytometer counts) are: immature, 168; mature, 170; and fertilized, 167. Thus it appears that the rate of oxygen consumption for the *Asterias* eggs is the same whether mature, immature, or fertilized. Their rate is of the same order of magnitude as that for the fertilized *Arbacia* eggs, and is five times that of the unfertilized (Tang, 1931). If we take into consideration the diameter of the unfertilized *Arbacia* eggs (74 micra) and that of the unfertilized *Asterias* eggs (160 micra), we obtain a ratio of 1 : 2.2. On squaring, it becomes 1 : 4.8, which is the ratio of the Qo_2, of these eggs, indicating that when expressed in terms of amount of oxygen consumed per unit surface, the Qo_2 of the two unfertilized eggs agree. Such a relation fails to hold in the case of the fertilized eggs.

I wish to express my sincere gratitude to Professors R. S. Lillie and R. W. Gerard for their advice and suggestions during the course of this study.

CITATIONS

Loeb, J., and H. Wasteneys, 1912. *Arch. entw.-mech. Organism.*, **35**: 555.
Tang, P. S., 1931. *Biol. Bull.*, **60**: 242.
Warburg, O., 1926. Über Stoffwechsel des Tümoren. Berlin. Julius Springer.

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NOTES ON THE FEEDING MECHANISM AND ON INTESTINAL RESPIRATION IN CHÆTOPTERUS VARIOPEDATUS[1]

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A healthy *Chætopterus* introduced into a glass tube rapidly lines this with a parchment-like secretion. One individual, after living in such a tube for two weeks, extended the lining beyond the aperture of the tube at one end. The prolongation was sharply constricted, showed successive thickened rings, and terminated in an expanded rim; it was, in fact, an exaggeration of the constriction at the end of a normal tube.

The tube current in such a preparation, as is well known, enters the tube anteriorly and leaves posteriorly, maintained by the rhythmic beating of the fans on segments 14, 15, and 16. It is weak ventral to the animal, but strong dorsally, and is directed under the arch formed by the long parapodia of segment 12.

This main tube current provides the food supply, the nature of which has been described by Enders (1909). The collecting mechanism has been described by several authors as follows: the broad ciliated buccal funnel collects directly from an extensive antero-ventral field; in addition, ciliated grooves on the dorsal side of the thorax collect from the tube current. To demonstrate this when the animal is removed from its tube, food particles must be supplied by dropping them onto the thorax from a pipette.

Such particles are collected in a mucoid stream into grooves along the inner edge of the arch formed by the parapodia of segment 12, and pass from this anteriorly in a median groove. Particles which happen to fall on the ventral face of the thorax are passed in laterally moving streams dorsally, between some of the posterior parapodia—being thus brought into the dorsal collecting field. The median dorsal groove does not lead directly into the mouth, but ends blindly in a dilatation posterior to the dorsal lip overhanging the mouth (Fig. 1). The wall round the terminal dilatation is thickened and raised, and forms a three-lobed prominence. The anatomical details of the structure of the groove have been given by Joyeux-Laffuie (1890).

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[1] The following notes were made during a visit to the Marine Biological Laboratory at Woods Hole during August and September, 1929. The author wishes to thank Dr. F. R. Lillie for his interest and assistance.

In describing the transference of food from the groove into the mouth, Enders stated that "the lip of the buccal funnel is drawn backwards, and the ciliary groove, which now extends beyond the dorsal border of the mouth, permits the granules to fall directly upon the ventral lip of the funnel." Described in more detail, the complete course of events is as follows.

While the food is passing forward in the groove, the anterior edge of the dorsal lip is reflected posteriorly until its tip comes into contact with the wall of the terminal dilatation of the groove (Fig. 2). To aid this, the posterior half of the lip is depressed by ventral muscular contractions centering in two areas. One of these is immediately anterior to the end of the groove; the other forms a pit within the tissue of the lip. These two contractions result in the formation of a deep transverse groove between the anterior end of the dorsal groove and the anterior edge of the lip, arched over by the lip when this is reflected.

A further contraction now follows, as a result of which the exposed surface of the reflected lip becomes depressed in the median-sagittal line so as to form a deep longitudinal groove, which is a direct continuation of the groove on the thorax; the food particles can now pass from one to the other without any interruption or obstruction (Fig. 3).

When the food has passed over the groove and into the mouth, the lip is relaxed and returns to its position of rest. If it happens that the food is removed before it reaches the anterior end of the thorax, the lip does not complete this normal cycle of action, but is relaxed at once.

The stimulus which excites this reflex is apparently the presence of solid particles in the food groove. In addition to this mechanical sensitivity there may be some sense of chemical discrimination also, as the animals often discard carmine or other non-nutritive particles.

The lip action can be induced experimentally in the following manner. A fine brush from which all but a few hairs have been removed is drawn slowly along the groove from the posterior end, and the lip responds as described above: the advantage of using such a type of stimulation is that one point only of the groove is stimulated at any one moment. While the brush is in the posterior end of the groove there is no response, but when it reaches approximately the level of the third or fourth setigerous parapodium, reflection of the lip begins. The exact extent of the anterior sensitive area varies, but it seems to be not more than one-fifth of the total length of the groove. The lip is reflected before the food reaches it,—it acts at such a time, in fact, that when the first granules reach the end of the thoracic groove, the groove on the lip is just ready to receive them. Stimulation of the anterior raised termination of the groove causes immediate response irrespective of whether or not the groove itself has been stimulated previously.

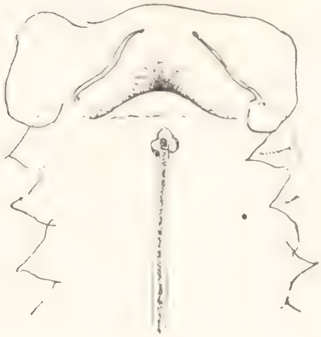

FIG. 1.

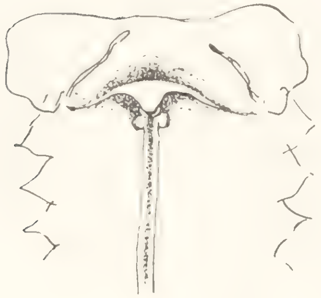

FIG. 2.

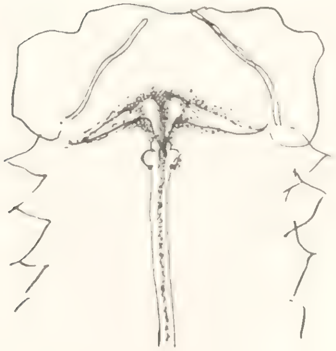

FIG. 3.

FIG. 1. Anterior end, dorsal view, showing lip in position of rest. (Diagrammatic.)

FIG. 2. The same, with lip reflected posteriorly.

FIG. 3. Lip reflected and grooved longitudinally ready to receive the food stream from the thoracic groove.

In connection with this reflex action it is of interest to compare a figure given by Joyeux-Laffuie of the nervous system of *Chætopterus.* He shows a pair of nerves arising from the dorsal region of the circum-œsophageal ring and extending over approximately the anterior half of the setigerous thoracic segments—thus corresponding more or less in their distribution with the extent of the sensitive area.

Observations on intestinal respiration were made on individuals which had recently regenerated some posterior segments. Such new somites are transparent and free from pigment, and are particularly favorable for this purpose..

Stephenson (1913) mentions *Chætopterus* in his paper on intestinal respiration and records an in-going current at the anus, but adds that no anti-peristaltic contraction of the gut was seen. Such contractions have, however, been seen repeatedly in recently regenerated somites, though not in normal pigmented individuals. In addition to anti-peristaltic contractions, the "gulping" action recorded by Stephenson in several genera was seen at times, and there was in some cases also observed a pulsating or pumping action in the gut at some distance in front of the anus.

The simple anti-peristaltic action will be described first. It is an anteriorly moving wave of contraction passing over the alimentary canal in the few hind somites, constricting both the walls and the lumen. The number of segments over which it persists varies, but it has repeatedly been watched over at least seven segments, and occasionally over one or two more. The interval of time separating successive waves varies also, both in different individuals, and in the same individual on different occasions: in fact, the activity often ceases altogether. When active, the waves may follow each other at intervals of 4, 3, 2, or even 1½ seconds.

A regular "gulping" action was seen only rarely, though it is not uncommon to see the anus opening and closing at irregular intervals; this action is usually associated with a movement of protrusion and retraction of the posterior end of the canal. In one particularly favorable individual the "gulping" action maintained a rhythm with intervals of approximately one second, while after every three or four gulps there was a pause while a peristaltic wave passed anteriorly over a few segments.

The pumping mechanism mentioned above probably serves to reinforce the peristaltic wave: it may synchronize with the wave, or may have an independent rhythm. It is seen less frequently than the peristalsis. The action occurs about seven somites in front of the hind end, but as details vary, a few precise examples will be given.

One individual examined had seven newly regenerated somites at the hind end, all perfectly colorless and transparent. Anti-peristaltic waves passed over the alimentary canal in the posterior segments, succeeding each other at intervals of approximately four seconds. At the same time, the gut in the fifth segment from the hind end maintained a pulsation independent of this wave, the beats occurring at intervals of about one second. In another case peristaltic waves passed forwards over the gut, and as they reached the seventh segment from the hind end and were becoming weak, they received renewed impetus and persisted through two or three segments further.

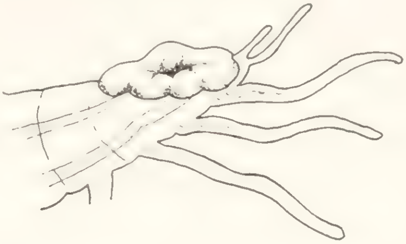

FIG. 4. Posterior end, showing the intestine protruded at anus.

The function of this anal and intestinal mechanism may be two-fold, as suggested by previous authors (see Stephenson, 1913 and 1930).

In the first place, the in-going anal current may be respiratory: *Chætopterus* has no special respiratory organs, and there are several features which support the suggestion that the anus may play a part in respiration. In the present case all the observations were made in aquaria, hence, although the aëration was maintained as efficiently as possible, it was not normal. However, it is known that in the natural situation the animal often protrudes its hind end from the tube. Further than this, there is a terminal swelling on the alimentary canal which is protrusible, and which, when everted, forms a rosette-shaped protrusion around the anus (Fig. 4). When retracted, the termination of the canal appears compressed and much folded. There is also, as described by Enders, a longitudinal groove in the intestine in which the cells are distinguished by their stronger cilia and by the absence of green granules. In the oligochætes similar grooves are associated with an in-going respiratory current, and the same explanation may perhaps be true here.

In the second place, the muscular activity of the intestinal wall may be for the purpose of propulsion of blood in a peri-enteric sinus or plexus: such a peri-enteric plexus exists in *Chætopterus* according to Probst (1929).

SUMMARY

1. The food of *Chætopterus* is transferred from the dorsal thoracic groove to the mouth by the temporary adaptation of the dorsal lip to form a conducting channel leading directly into the mouth from the blind anterior termination of the groove.

2. This reaction of the dorsal lip can be induced by mechanical stimulation of the anterior part of the dorsal groove.

3. Clear and colorless somites which have been regenerated recently at the hind end of a *Chætopterus* demonstrate the occurrence of antiperistaltic contractions in the alimentary canal of the hind segments; such individuals also show a " gulping " action at the anus, and an accessory pumping mechanism in the walls of the intestine amplifying the peristaltic contractions.

BIBLIOGRAPHY

ENDERS, H. E., 1909. A Study of the Life History and Habits of Chætopterus variopedatus, Rénier et Claparède. *Jour. Morph.*, **20**: 479.

JOYEUX-LAFFUIE, J., 1890. Étude monographique du Chétoptère (Chætopterus variopedatus, Rénier). *Arch. Zoöl. Expér.*, sér. 2, **8**: 245.

PROBST, G., 1929. Das Blutgefässystem von Chætopterus variopedatus Rénier. *Pub. Stat. Zoöl. Napoli,* **9**: 317.

STEPHENSON, J., 1913. On Intestinal Respiration in Annelids; with Considerations on the Origin and Evolution of the Vascular System in That Group. *Trans. Roy. Soc. Edin.,* **49**: 735.

STEPHENSON, J., 1930. The Oligochæta. Oxford University Press.

DIPLOID MALE PARTS IN GYNANDROMORPHS OF HABROBRACON

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There are many theories of the origin of male parts of gynandromorphs in Hymenoptera. They may be classified under three headings,—gynogenetic, androgenetic, and biparental.

The gynogenetic theories presuppose egg binuclearity. Male parts arise from a blastomere nucleus (Boveri, 1915), a separate oögonial nucleus (Dönhoff, 1860; Doncaster, 1914), or a second oötid from the same oöcyte (Whiting, P. W., 1924). According to these theories male parts should show only maternal characters.

The theory of androgenesis involves polyspermy (Morgan, 1905). The supernumerary sperm nucleus undergoes cleavage resulting in haploid male tissue. Male parts then should show only paternal characters.

The biparental theory that holds for the majority of gynandromorphs in *Drosophila* has been applied by Morgan to the bee. According to this theory, the gynandromorph starts out as a female but loses an X-chromosome in an early embryonic stage. The resulting one X tissue is male. Male tissue would then be biparental in inheritance of autosomal traits, but as regards sex-linked traits there is an equal chance that male tissue would be of paternal or of maternal origin according to which X-chromosome was lost.

Another biparental theory assumes both egg binuclearity and polyspermy. Fertilization of the two egg nuclei by separate sperm may result in tissues of opposite sex depending upon the chromosome composition of the two zygotes. In case of female digametism, male tissue would be entirely biparental but in case of male digametism, male tissue would be biparental for autosomal traits, matroclinous for sex-linked characters.

Previously published records of gynandromorphs in *Habrobracon* have made it seem highly probable that male tissue is gynogenetic in this form. A single case (No. 325) was, however, reported in which male parts were patroclinous (Whiting, P. W., 1928). This example had clearly male head and ocelli of male size, which as well as the eyes were black and of paternal origin since the mother had recessive ivory.

A second instance came to light in August, 1930, when Mr. Hurst Shoemaker was studying progeny from crosses of females from an orange-eyed, *o*, defective-veined, *d*, stock (No. 3) with males of type stock No. 1. Among the type females and orange-defective males expected from this cross, there was found a gynandromorph (No. 438) with male head, black eyes and black ocelli. The ocelli were of typical male size set in a dark area of male character. The antennæ were also male and the instincts were in general male; for it attempted to mate with females and was indifferent to host caterpillars, except for a slight momentary reversal when it attempted to use its sting against a caterpillar. The abdomen was entirely female, body pigment and wings symmetrical. The primary wings were of normal venation, a patroclinous trait but sex of wings in this instance, presumably female, could not be accurately determined.

Further evidence has been obtained in regard to the nature of gynandromorphs which bears upon the theories above presented. The following summary involves only those with parents bearing diverse traits,[1] so that character of male structures is decisive as regards origin.

Of gynandromorphs from mothers carrying the dominant factor there were four in which male parts were matroclinous. These are decisive against the androgenetic theory for this case, but do not preclude a biparental origin.

Of gynandromorphs from mothers carrying the recessive factor there are 38 in which male parts were matroclinous. Among these the total number of matroclinous traits in male parts is 50. These instances are not only contrary to the androgenetic theory but against the biparental theories as well.

The significance of the two individuals with male parts patroclinous from recessive mothers will be discussed below.

Female parts of sex mosaics have generally been regarded as biparental, and should accordingly show the dominant traits of either or both parents. Four gynandromorphs obtained from mothers with a dominant factor have shown this dominant. Twenty-eight obtained from mothers with one or more recessive factors have shown 33 dominant patroclinous traits, each dependent upon a single genic difference. Evidence is entirely in agreement with biparental origin of female parts.

The reason for the excess of gynandromorphs from recessive mothers and dominant fathers over those from the reciprocal is merely that many more crosses are made in which the female bears the recessive. There is no greater tendency for recessive females to produce them. Females bearing one or more recessives are used in connection with investigations on biparental males.

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[1] Many of the mutant factors causing these traits arose in the course of X-radiation experiments conducted under a grant from the Committee on Effects of Radiation on Living Organisms, National Research Council.

Crosses of certain stocks regularly produce a few males resembling their sisters in showing the dominant traits of both parents (Whiting, Anna R., 1927). These have been called at various times, anomalous, patroclinous, biparental, or diploid males. Evidence has been gradually accumulated which indicates their diploidism. It is perhaps useless to speculate at this time as to why they are males if diploid; but it has been shown that occurrence of these males is dependent upon the stock of mother as well as of father. Thus stock No. 3 female by related No. 1 male produces biparental males while the same female by unrelated No. 11 male fails to produce them. No. 11 males may, however, sire biparental sons when crossed with related No. 12 females. It is suggested that absence of an X-chromosome either from the reduced egg or from the sperm may be the determining factor, but for this there is as yet no evidence.

The two gynandromorphs with male parts patroclinous may be explained by the theory of loss of an X-chromosome in development but, since both came from crosses producing biparental males, they are regarded as having developed from binucleate eggs in which each nucleus was fertilized by a different sperm. Egg binuclearity and dispermy are both involved with absence of an X-chromosome either from one egg nucleus or from one sperm nucleus.

LITERATURE CITED

Boveri, Th., 1915. Über die Entstehung der Eugsterschen Zwitterbienen. *Arch. f. Entw. Organism.*, 41: 264.

Doncaster, L., 1914. On the Relations between Chromosomes, Sex-limited Transmission, and Sex-determination in *Abraxas grossulariata*. *Jour. Genetics*, 4: 1.

Dönhoff, 1860. *Beitr. z. Bienenkunde.* I. Über Zwitterbienen. Bienenzeitung.

Morgan, T. H., 1905. An Alternative Interpretation of the Origin of Gynandromorphous Insects. *Science*, 21: 632.

Whiting, Anna R., 1927. Genetic Evidence for Diploid Males in Habrobracon. *Biol. Bull.*, 53: 438.

Whiting, P. W., 1924. Some Anomalies in Habrobracon and their Bearing on Maturation, Fertilization, and Cleavage. Am. Soc. Zoöl. Abstr. 140, *Anat. Rec.*, 29: 146.

Whiting, P. W., 1928. Mosaicism and Mutation in Habrobracon. *Biol. Bull.*, 54: 289.

Whiting, P. W., and Anna R. Whiting, 1927. Gynandromorphs and Other Irregular Types in Habrobracon. *Biol. Bull.*, 52: 89.

A GYNANDROMORPH OF HABROBRACON FROM A POST–REDUCED BINUCLEATE EGG[1]

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The origin of gynandromorphs from binucleate eggs has been established genetically for various insects. Boveri (Boveri, Th., 1915) regarded the two nuclei as resulting from a first cleavage division of a reduced egg nucleus with consequent equality of maternal contribution to male and female parts of the resulting embryo. His theory may be called post-maturational.

Whiting (Whiting, P. W., 1924) interpreted the origin of a haploid mosaic male from a heterozygous mother, *oDwM/OdWm,* as due to pre-reduction of *Dd* and *Ww,* post-reduction of *Oo* and *Mm.* The two cleavage nuclei would then be products of the second oöcyte division, one corresponding to the reduced egg nucleus, the other to the second polar body. This maturational theory was later (Whiting, P. W., and Whiting, Anna R., 1927, and Whiting, P. W., 1928) applied to the origin of gynandromorphs. Contrary to the view of Boveri, the two oötid nuclei may bear different genes for those loci undergoing post-reduction.

Other theories, maturational and pre-maturational, have been advanced by various authors allowing difference of maternal contribution. Goldschmidt (Goldschmidt, R., 1931) has genetic evidence for the existence of such differences in the silkworm, *Bombyx,* as well as cytological results favoring Whiting's maturation theory.

There is now abundant genetic evidence in *Habrobacon* for the existence of differences in the maternal contribution to the genetically different parts of haploid mosaic males from heterozygous virgin mothers. It has been supposed that gynandromorphs have an origin similar to these males except that one of the oötid nuclei is fertilized and that consequently female parts are diploid and biparental, while male parts are haploid and matroclinous. There has been, however, up to the present time no critical case in this wasp contrary to Boveri's scheme.

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[1] The gynandromorph discussed in this paper was found during the course of experiments conducted under a grant from the Committee on Effects of Radiation on Living Organisms, National Research Council.

In the course of experiments at the Marine Biological Laboratory, Woods Hole, during the summer of 1931, a number of females (stock No. 3), homozygous for the recessive genes for orange eyes, *o,* and defective wing venation, *d,* were crossed to type (black-eyed, *O,* normal-winged, *D*) males (stock No. 1). When the diheterozygous, *OoDd,* type daughters from this cross were bred, the occurrence of females among their progeny indicated that they had mated with their orange-defective brothers. In addition to the four classes of males and of females expected,—type, orange, defective and orange defective,—there appeared in one fraternity a gynandromorph, No. 513 (Fig. 1).

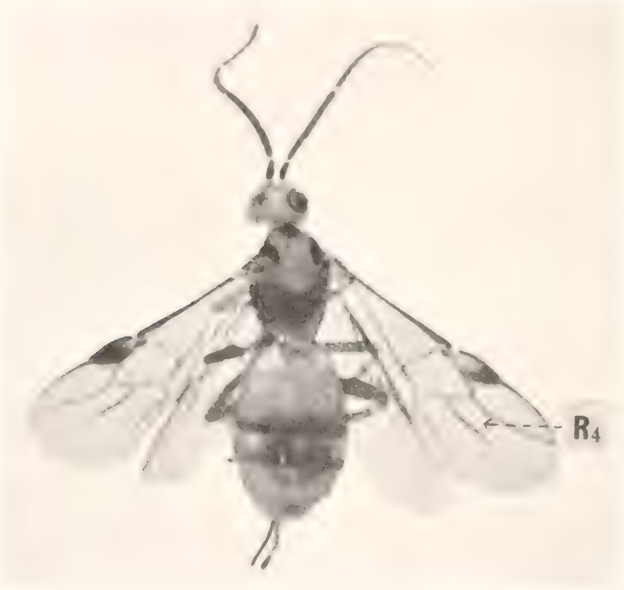

Fig. 1. × 21.

The antennæ were male; the left having 21 segments more or less deficient terminally, the right having 23 of normal appearance. Both eyes were orange in anterior and dorsal regions, black in posterior and ventral. The ocelli (Fig. 2) were small and therefore female, the lateral orange, the median containing some dark pigment. The area between the median and right ocelli was dark while that around the left was yellow. The dark area may be presumed to be male in constitution, as male integument tends to be darker than that of the female under similar conditions of temperature, etc. The fact that the ocelli were orange and female indicates that the orange parts of the compound eyes as well were female and the black parts male. The left primary wing was smaller, therefore presumably male, and showed defective venation, the fourth branch of the radius (R_4) being completely lacking, while the right primary was larger (female) and type. The secondary wings also showed the sex difference in size. In the prosterna the left side showed the darker (male) pigmentation. The abdomen was female throughout.

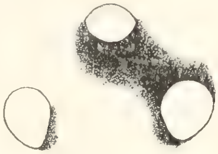

FIG. 2. × 160.

The origin of this gynandromorph may be represented by the following formula:

$$\begin{array}{rl} \text{First polar body} & OoDd \\ \hline \text{Oötids} & Od\,|\,oD \\ \text{Sperm nucleus} & \underbrace{\,|\,od}_{\text{Cleavage nuclei}} \end{array}$$

The fact has been well established for *Habrobracon* that the mother contributes to both male and female parts of gynandromorphs. The circumstances of this case, in which the paternal genes were recessive for the loci concerned, allowing the dominant genes of the mother to express themselves, indicate that the maternal contributions to the male and female parts of this gynandromorph are different. That direct evidence of this sort, in favor of the hypothesis of Goldschmidt and Whiting, has not been found before in *Habrobracon* may be attributed to the fact that most of the crosses are made with homozygous females, and that in the few gynandromorphs reported from heterozygous mothers, the distribution of the haploid and diploid tissues did not permit differences between the maternal contributions to show. By making enough appropriate crosses with females heterozygous for factors affecting various parts of the body, it should be possible to produce gynandromorphs giving further evidence of the same sort.

LITERATURE CITED

BOVERI, TH., 1915. Über die Entstehung der Eugsterschen Zwitterbienen. *Arch. f. Entw. Organismen,* 41: 264.

GOLDSCHMIDT, R., 1931. Die Sexuellen Zwischenstufen. Julius Springer, Berlin. Pages 437–445.

WHITING, P. W., 1924. Some Anomalies in Habrobracon and their Bearing on Maturation, Fertilization and Cleavage. *Anat. Rec.*, **29**: 146.

WHITING, P. W., 1928. Mosaicism and Mutation in Habrobracon. *Biol. Bull.*, **54**: 289.

WHITING, P. W., AND ANNA R. WHITING, 1927. Gynandromorphs and other Irregular Types in Habrobracon. *Biol. Bull.*, **52**: 89.

ON CERTAIN PHYSIOLOGICAL DIFFERENCES BETWEEN DIFFERENT PREPARATIONS OF SO-CALLED "CHEMICALLY PURE" SODIUM CHLORIDE

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I

It is the purpose of the present paper to direct the attention of biologists to important differences in the toxicity to living cells and organisms of certain commercial brands of so-called C.P. sodium chloride which have usually been treated in the past as being more or less identical chemically. The brands in question have all been used frequently at the Marine Biological Laboratory and other scientific institutions in this country; and, in view of the striking differences that will be shown to exist between them, the question arises how far the work of different investigators, who have in the past used sodium chloride of unspecified origin, is comparable and, indeed, how far many published statements concerning the physiological properties of this salt in pure solutions may be generally true. While these questions cannot as yet be answered with entire certainty, the necessity is clearly indicated for much greater care in the future than has been exercised in the past in physiological work involving this commonest of all salts.

The observations which formed the beginning of this investigation were made more or less accidentally in connection with certain unpublished studies on the hemolytic effects of ammonium chloride on the erythrocytes of the various classes of vertebrates, particularly the fishes. In the course of these studies, controls of isotonic NaCl were used for comparison, the salt employed being that which happened at the time to be in general use at the Marine Biological Laboratory. It soon became apparent that whereas the erythrocytes of the mammals remained intact almost indefinitely in such control solutions, those of several species of fishes, among them the sea robin, the butterfish, the cunner, the tautog, the mackerel, the scup and the fresh water perch, underwent destruction in times ranging from a few minutes to several hours, though failing to do so in similar solutions of KCl or $CaCl_2$ or in properly diluted sea water.

The unique behavior of NaCl is brought out in Fig. 1, in which are plotted against the times in hours from the beginning of the experiment the cell counts, obtained by the usual hemocytometer method, of suspensions of the erythrocytes of the sea robin (*Prionotus carolinus*) in approximately isotonic solutions of KCl, NaCl and $CaCl_2$ and in a physiologically balanced mixture of the three salts. The rapid destruction of the erythrocytes here shown in solutions containing NaCl and their preservation in the other solutions are entirely typical of dozens of experiments made during the summer of 1926 with the particular brand of salt in question, not only on the erythrocytes of the sea robin but on those of the other species mentioned above as well.

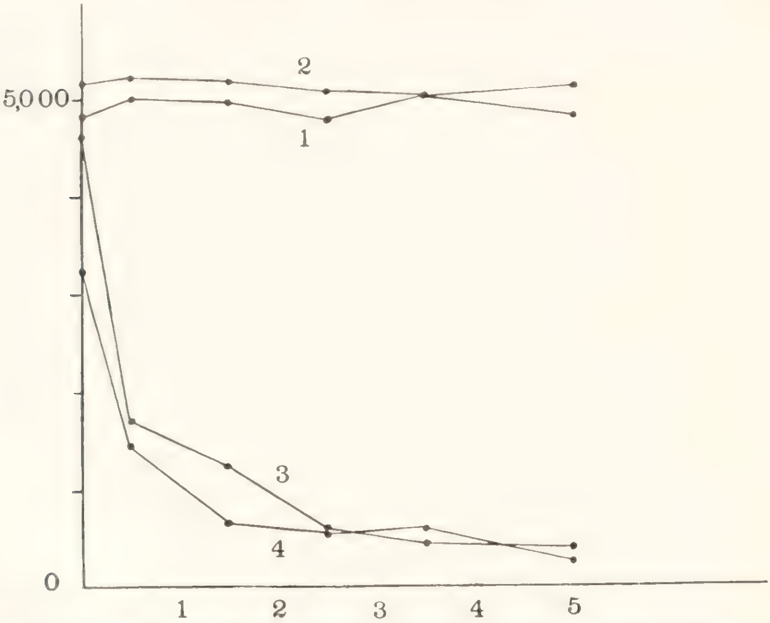

FIG. 1. Effect of exposing erythrocytes of the sea robin (*Prionotus carolinus*) to: (1) M/3.7 KCl, (2) M/5.5 $CaCl_2$, (3) M/3.7 NaCl and (4) a mixture of these solutions in the proportions of 2 : 2 : 96. Ordinates represent numbers of cells per cubic millimeter and abscissæ times in hours.

On repeating the experiments the following year our surprise was great when the expected hemolysis in NaCl solutions completely failed to appear, the erythrocytes remaining intact in such solutions for many hours with no more evidence of injury than when KCl or properly diluted sea water was employed. The only difference between the two sets of experiments was that by chance a new brand of C. P. NaCl had been substituted in 1927 for that used in 1926. On going back to the former brand the earlier results could again be repeated at will. Evidently there was in respect to their hemolytic properties at least, a very decided difference between two preparations of NaCl, both presumably of good quality and both in common use at the Marine Biological Laboratory and elsewhere. Because of the possible importance of such differences in physiological work, further experiments on fish erythrocytes were therefore undertaken with the more common commercial brands of C.P. sodium chloride; and the results were later extended to several other types of living material. The general outcome of these experiments may now be described.

II

In all, five brands of C.P. NaCl, each prepared by a different manufacturer, were studied. In every case, samples from several separate and previously unopened containers were used. In order to avoid any possibly unjust conclusions being drawn as to the relative values of the salts of the different manufacturers for the chemical purposes for which they were primarily intended, the different brands will be designated merely by the letters *A* to *E*, inclusive. It is perhaps not improper to say that the brand designated by *A*, which is the least harmful to fish erythrocytes of all those studied, being in fact practically as harmless as KCl, is the Kahlbaum salt of the best quality obtainable. Of the other four brands, *B* was at times almost as good as the Kahlbaum preparation, but at other times was distinctly harmful, the differences observed depending partly on the lot of salt used and especially on the species of fish furnishing the erythrocytes. In our earlier experiments, in which the decidedly resistant erythrocytes of the sea robin were employed, this brand was almost indistinguishable from *A*, but in later observations made by Dr. A. K. Parpart, working with one of the authors on another problem, it appeared that it was quite incapable of preserving for any length of time the much less resistant erythrocytes of the tautog and the cunner which were, however, not markedly injured by brand *A*. Brands *D* and *E* were invariably destructive to all the fish erythrocytes studied, though more rapidly so to some than to others. Brand *C*, as far as it was studied, appeared to be relatively harmless, but our information about it is not very complete.

A typical experiment in which the effects on the erythrocytes of the scup (*Stenotomus chrysops*) of brands *B*, *C*, *D* and *E* and of KCl is illustrated in Fig. 2. The blood in this case, as in all others here reported, was freshly obtained from a living fish without the use of any anticoagulant and was added immediately to the solutions in question in the proportion of approximately 1 to 200 by volume (*i.e.*, one drop to 10 cc.). A slight variation in the sizes of the drops of blood was of no significance, since cell counts were made in every case. In the absence of exact information concerning the osmotic pressures of the various bloods studied, the concentration of NaCl employed was taken, unless otherwise indicated, as 0.25 M. Such solutions have a freezing point of approximately −0.86° C., which is not very far removed from that of the plasma of the various marine teleost fishes for which figures are available; and at all events the concentration was the same for the various brands of salt employed, so that the results were entirely comparable among themselves.

It will be noted in Fig. 2 that for the duration of the experiment (5 hours) there was no appreciable decrease in the number of erythrocytes in solutions of brands *B* and *C*, while in similar solutions of brands *D* and *E* the numbers had decreased very appreciably within one half hour and very few erythrocytes remained at the end of four hours. It may be mentioned incidentally that the erythrocytes of the scup, like those of the sea robin, are relatively resistant ones; those of the butterfish or of the cunner disappear far more rapidly.

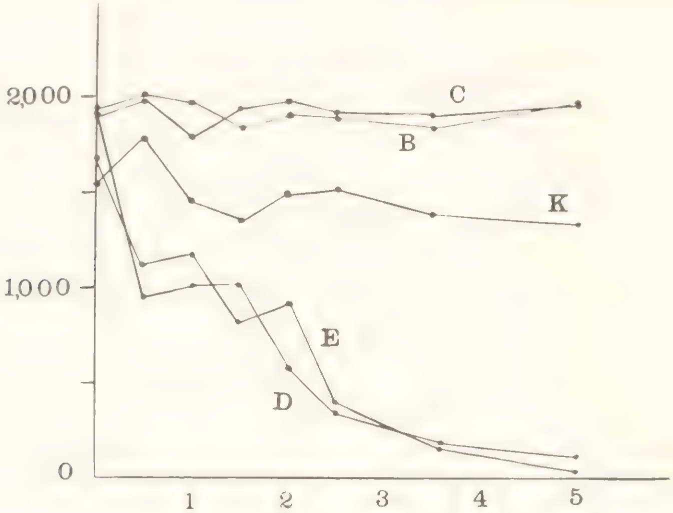

FIG. 2. Effect of exposing erythrocytes of the scup (*Stenotomus chrysops*) to M/4 solutions of brands *B*, *C*, *D* and *E* of NaCl and to M/4 KCl. Ordinates represent numbers of cells per cubic millimeter and abscissæ times in hours.

This particular experiment is typical of several dozen others differing in detail but all giving essentially similar results. In addition, many incidental observations by W. A. Smith, S. E. Hill and A. K. Parpart working with one of the authors on other problems in which cell counts were not made but hemolysis was followed by a macroscopic method have been in entire agreement with the results pictured in Fig. 2. It may, therefore, be considered as definitely established that the erythrocytes of certain fishes are affected in an entirely different manner by various preparations of C.P. NaCl in common use.

III

As to the cause of these differences, two main possibilities suggest themselves: either pure sodium chloride is in itself destructive to the erythrocytes and its harmful effect is antagonized by impurities of some sort present in brands *A* and usually in brands *B* and *C*, or pure sodium chloride is in itself relatively harmless to this form of material and the injury is due to a toxic impurity of some sort in brands *D* and *E* and sometimes in *B* and *C*. Though the first type of explanation might perhaps appear to be somewhat far-fetched, it must not be forgotten that pure NaCl has been generally considered to be highly toxic (Loeb, 1900; Osterhout, 1922) and that its toxic effects may be antagonized by very low concentrations of plurivalent cations—for example, in the case of the cilia of *Mytilus*, according to Lillie (1906), M/51,200 $FeCl_3$ is strikingly antitoxic.

TABLE I

Effect on erythrocytes of the freshwater perch of solutions of NaCl of brands B and E before and after recrystallization. The figures represent numbers of erythrocytes in 1 cubic millimeter of a dilute suspension.

Number of Experiment	Brand B				Brand D			
	Original Salt		Recrystallized Salt		Original Salt		Recrystallized Salt	
	Beginning of Experiment	After 1 hour	Beginning of Experiment	After 1 hour	Beginning of Experiment	After 1 hour	Beginning of Experiment	After 1 hour
1	245	200	200	190	225	0	240	0
2	230	225	225	205	175	0	150	0
3	200	250	250	160	150	0	150	0
Average	225	225	225	218	183	0	180	0

It was thought that some light might be thrown upon these two alternative types of explanation by a comparison of the effects upon the same material of a harmful and a harmless brand of salt before and after recrystallization. Brand *B* was known, for example, to be harmless and brand *E* to be highly destructive to the erythrocytes of the freshwater perch. If the first of the two types of explanation were correct, recrystallization should tend to make brand *B* more toxic than before and leave *E* unchanged; if, on the other hand, the second were correct, recrystallization should make *E* less toxic and leave *B* unchanged.

Lack of time prevented extensive recrystallizations from being carried out, but in Table I are represented the results of one experiment in triplicate of this sort. Because of the difference in the osmotic pressure of the blood of freshwater as compared with marine teleosts, the concentration of NaCl here employed was 0.147 M, which has a freezing point in the vicinity of that found by Garrey (1916) for the blood of a number of freshwater fishes, *i.e.*, approximately −0.50° C. It will be noted that the experiment shows no significant change in the properties of either salt after recrystallization. It is therefore inconclusive, so far as throwing light upon the nature of the differences in the physiological properties of the salt preparations in question is concerned, but it does indicate one fact of great practical importance, namely, that any impurities that may be present are difficult to remove by recrystallization.

The question of possible antagonism was more directly and extensively attacked in another way. Since it is well known from the work of Loeb and others that perhaps the most effective single antagonist of the toxic effects of sodium is calcium, and that solutions containing sodium, calcium and potassium in the proper proportions form for most cells and tissues a fairly good substitute for their natural medium, attempts were made to find some combination of the chlorides of calcium or of calcium and potassium, with the toxic brands of sodium chloride that would remove or at least greatly diminish the hemolytic effect of the latter. In this we were completely unsuccessful. In particular, the addition to the toxic brands of NaCl of $CaCl_2$ and KCl in the approximate proportions in which they occur in the body fluids of the vertebrates or in sea water was almost without effect (see Fig. 1). Only when isotonic solutions of $CaCl_2$ or KCl or both were added to similar solutions of NaCl in sufficient quantities to dilute the latter appreciably did a diminution of the hemolytic effect become apparent. This effect, however, which is entirely different from antagonism, is what would be expected if the NaCl carried a toxic impurity.

It has been mentioned above that the erythrocytes of marine fishes are preserved fairly normally in properly diluted sea water, which is a well-known example of a physiologically balanced salt mixture. In several experiments, diluted sea water was mixed in different proportions with approximately isotonic solutions of one of the toxic brands of NaCl. In such experiments it was found that the hemolytic effect of the added salt could, in general, be detected to an extent that depended upon its concentration in the mixture. This result is again what would be expected if a toxic impurity were associated with the sodium chloride.

In view of the fact that all attempts to demonstrate a physiological antagonism between the toxic brands of NaCl and various calcium and potassium mixtures failed completely, the view was definitely abandoned that hemolysis by some salt preparations is due to the destructive effects of pure NaCl itself. The fact that brand *B* could manifest its harmful effects even in the presence of a considerable excess of diluted sea water and the additional fact that brand *A*, which has been consistently harmless, is at the same time one generally considered by chemists to be of especially high purity seem to point rather to something added to the sodium chloride in the toxic samples of the salt. It may be mentioned that Dr. Eric G. Ball has recently obtained evidence of a very direct and convincing nature that the hemolytic effects of some brands of NaCl are due to contained impurities. This evidence will soon be published elsewhere.

Accepting the view that some brands of C.P. NaCl contain an impurity highly destructive to the erythrocytes of fishes there may be mentioned briefly several of our unsuccessful attempts to determine the nature of this impurity. Partly because our results on this point were completely negative and partly because of the much more extensive observations along the same lines soon to be published by Dr. Ball, it will be sufficient here merely to eliminate from further consideration one or two conceivable factors.

It is known that the erythrocyte is, in general, fairly sensitive to pH changes and also that some preparations of so-called "neutral salts" are not entirely neutral. One of the first of the possibilities to be considered, therefore, was the reaction of the various solutions studied. It was found that as far as pH measurements can be made upon completely unbuffered solutions there were no significant differences in reaction between the different sodium chloride solutions and the distilled water used to make them up, or between these solutions and similar ones of completely harmless KCl. Furthermore, in one experiment there was added to solutions of brands *B* and *E* sodium bicarbonate in the proportion of one part of M/4 bicarbonate to twenty of M/4 NaCl. The pH of the resulting mixtures was then adjusted to 7.0 in each case by the addition of carbon dioxide in the proper amounts, a procedure which leaves the effective osmotic pressure of the mixture for the erythrocyte unaltered. Blood was then added to these well buffered mixtures, which were kept tightly stoppered throughout the remainder of the experiment. In spite of this careful regulation of the pH of the solutions, the erythrocytes underwent destruction in the presence of NaCl of brand *E* and remained intact in the case of brand *B* exactly as before. In still other experiments, it was shown that with a given it a change in the reaction of the solutions of two pH units (*i.e.*, from pH 6.0 to 8.0), which greatly exceeds any differences that could conceivably have been present in any of our experiments, had negligible effects upon the characteristic properties of the salts. It may be considered fairly certain, therefore, that the physiological differences between the salts in question are not due to pH effects.

In our search for possible impurities in sodium chloride preparations it was suggested to us by a chemical colleague that fluorides, which are fairly toxic to some living cells, might perhaps be concerned. Experiments were therefore made in which sodium fluoride was added in different proportions to the harmless salt of brand *B*. The proportions used ranged from a maximum concentration of NaF of 0.025 M by a series of dilutions with a factor of one fifth to a minimum concentration of the order of 0.00000001M. In none of these solutions, however, was brand *B* caused to resemble even remotely brands *D* and *E*, and it was therefore concluded that fluorides could scarcely be the impurity concerned. Similar experiments were carried out with salts of several toxic metals such as Pb, Hg and Cu which might conceivably have been present in traces in the more injurious salt preparations, but our results were again essentially negative.

As far as it was possible to carry our experiments up to the time when it became necessary to discontinue them in 1928, absolutely no clue had been obtained as to the nature of the hypothetical impurity. It should be emphasized, however, that a lack of knowledge of the nature of this impurity in no wise detracts from its physiological importance or renders its disturbing effects in certain types of experimental work less real.

IV

After establishing the fact that certain brands of so-called C.P. NaCl are highly destructive to the erythrocytes of a number of teleost fishes, experiments were undertaken to determine how far similar effects could be obtained with other forms of living material. It is evident that effects of this sort might, if unrecognized, cause considerable confusion in physiological work, particularly since all the brands of sodium chloride in question have been commonly used in such work—frequently with no published statements by which they may be identified. Additional experiments were therefore undertaken upon the following forms of material: mammalian erythrocytes, newly-hatched *Fundulus*, the eggs of *Arbacia* and the cilia of *Mytilus*. These experiments may be briefly described in the order mentioned.

As contrasted with the erythrocytes of the fishes, those of the mammals appear to be little injured by any of the brands of sodium chloride in question. It is doubtless owing to the comparative insensitiveness of this much-studied type of cell that the striking physiological differences in the properties of different salt preparations did not long ago become generally known. Our experiments were carried out on the blood of man, the ox, the dog, the cat, and the porpoise in the manner described above, the only difference in technique being that the concentration of the salt employed with the mammalian erythrocytes was 0.154M instead of 0.25M.

The results in the case of every mammal studied were, briefly, that for 10 or more hours at room temperature or for 24 hours partly at room temperature and partly in a refrigerator there was no appreciable hemolysis in any of the solutions. In experiments of longer duration, there were in a few cases some slight indications of differences in the expected direction, but these were so small and irregular as to be of little significance. It is possible that by employing aseptic precautions, which were not practicable in our experiments, and by keeping the erythrocyte suspensions for several days, constant differences might be demonstrated. For practical purposes, however, in ordinary experiments of short duration with mammalian blood it would appear to make little difference which brand of sodium chloride is used.

The experiments made upon *Fundulus heteroclitus* are of interest because it was upon this material that Loeb (1900 and later papers) obtained his most striking evidence of the toxicity of pure sodium chloride. Though for a number of reasons it appeared to be impossible that the effects described by Loeb could have been due to an impurity in the salt used rather than to the salt itself, it nevertheless seemed of some importance to determine whether with *Fundulus* the primary toxicity of pure sodium chloride might be modified in any way by the contaminating impurity supposed to be present in some preparations. The general result of our experiments was to show that this is, in fact, the case.

A typical experiment on newly-hatched, free-swimming fish is described in Fig. 3. In it, brands *B* and *E* were compared with respect to their ability to stop (*a*) the swimming movements of the animals and (*b*) the heart-beat. The concentration was in each case M/2, which is approximately isosmotic with Woods Hole sea water. It will be observed that the differences between the two salts are rather striking. At the end of 6 hours nearly all swimming movements had ceased in the animals exposed to brand *E*, while only a few of the individuals exposed to brand *B* had been similarly affected; some continued to move in this solution for over 12 hours. The cessation of the heart-beat also occurred much more rapidly in the presence of brand *E* than in that of brand *B*. These differences were observed many times with no exceptions. It may be concluded, therefore, that the observed effects of sodium chloride upon *Fundulus* depend to a considerable extent on the particular salt preparation employed.

A very sensitive test object for many purposes is the egg of *Arbacia*, whose rate of cleavage is affected in a readily measurable manner by very slight changes in, for example, the osmotic pressure and the carbon dioxide tension of the surrounding medium. Since pure isotonic sodium chloride is known to be toxic to this egg, it was thought that differences in the properties of different salt preparations might be shown by exposing fertilized eggs of *Arbacia* to them for suitable times and then determining the effect of such exposures upon the rate or the final percentage of cleavage. This was done in two ways: first by placing the eggs in the sodium chloride solution to be tested shortly before cleavage and allowing them to remain in the solution, and second by employing a short temporary exposure to the salt followed by a return to sea water. The first type of experiment proved to be entirely unsuitable owing to the failure of the eggs to divide at all, but the second type yielded results which, while not wholly satisfactory, were at least suggestive.

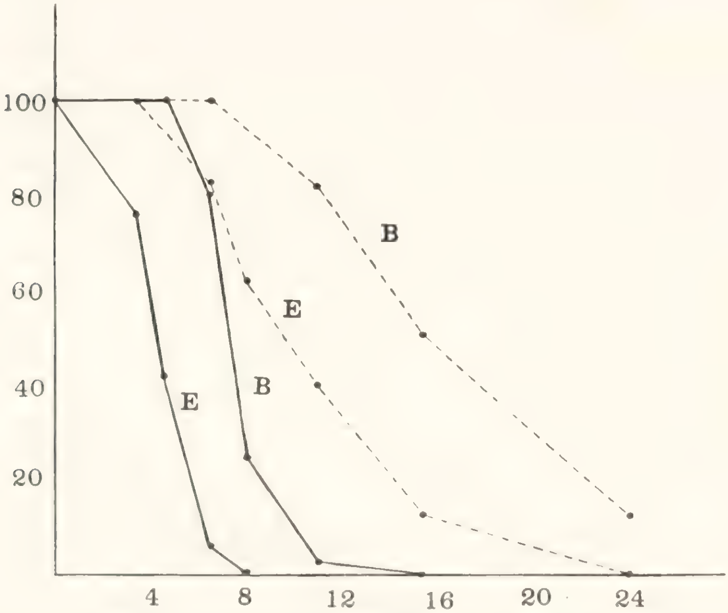

FIG. 3. Effect on newly-hatched *Fundulus* of M/2 NaCl of Brands *B* and *E*. Ordinates represent percentages of the animals showing swimming movements (solid lines) and heart-beat (broken lines); abscissæ represent times in hours.

The general result obtained from experiments of this latter type was that in some cases there were no very significant differences between the effects of salts *B* and *E*, but that in several cases where decided differences appeared, these were always in such a direction as to indicate a greater toxicity of brand *E* than of brand *B*. The reverse condition was never obtained. An experiment showing a very considerable difference in the toxicity of salts of brands *B* and *E* is summarized in Table II. It may scarcely be considered a typical experiment, however, since the differences observed were usually not so great.

Finally, a few observations were made upon the cilia of *Mytilus*, which Lillie (1906) has shown to be very rapidly injured in solutions of pure isotonic sodium chloride. It is, of course, difficult to treat the beat of cilia in a strictly quantitative manner, since different groups come to rest at different times and even within the same group certain individual cilia continue to beat long after the others have ceased to do so. It is possible, therefore, for the experimenter merely to estimate in a general way when some given end-point has been reached. As far as such estimates could be made, our experiments showed no significant differences between the different brands of sodium chloride, perhaps because in this case the pure salt itself is extremely toxic. For these particular experiments brand *A* was not available, but a sample of *B* of very low toxicity to fish erythrocytes was compared with brand *E* of high toxicity. The times for the attainment of the same estimated end-point with different gill filaments were found to be 15, 13, 4.5, 11 and 9.5 minutes (average 10.6 minutes) with brand *B;* and 11, 13, 7 and 10.5 minutes (average 10.4 minutes) with brand *E*, respectively. It is not impossible that more extensive and refined experiments would be capable of demonstrating definite differences, but as far as the present evidence goes, these would not likely be very great.

TABLE II

Effect on Subsequent Cleavage of Exposure of Fertilized Arbacia Eggs to Two Brands of Sodium Chloride

Length of Exposure	Percentage Undergoing Cleavage within 2 Hours	
	Brand B	Brand E
minutes		
5	68	3
10	39	4
15	4	1
20	2	5
25	8	2

Summarizing the results of the various experiments with different types of living material, it may be said that no significant differences between the different brands of sodium chloride studied have been found with mammalian erythrocytes or with the cilia of *Mytilus;* occasional but by no means constant differences in the expected direction have been found with the eggs of *Arbacia*, constant differences of considerable magnitude in the same direction occur with newly-hatched *Fundulus;* and differences of the most striking and characteristic sort are invariably present in the case of the material which was first studied, namely, the erythrocytes of certain fishes. Though up to the present time the fish erythrocyte is the most sensitive form of material known, it is not impossible that other types will be discovered in the future of even greater sensitivity. In the meantime, physiologists should constantly be on their guard, when working with sodium chloride, against what, at its worst, is capable of being a source of serious experimental errors.

Summary

1. Of five commercial brands of C.P. sodium chloride that have been studied, one is apparently always harmless and two always destructive to the erythrocytes of certain teleost fishes; one and perhaps both of the others are intermediate and somewhat more variable in their properties.

2. There is indirect evidence that the destructive effect of the toxic brands is due to the presence of an impurity of some sort, which has, however, not been identified. It is not removed by a single recrystallization of the salt.

3. Similar though much less striking differences have been found in the physiological action of the brands of sodium chloride in question upon newly-hatched *Fundulus* and less certainly upon the eggs of *Arbacia*. No constant differences have been noted in the case of mammalian erythrocytes or in that of the cilia of *Mytilus*.

4. It is suggested that in all physiological work in which sodium chloride is used particular attention should be given to the possibility of errors resulting from the presence in the salt of unknown toxic impurities.

BIBLIOGRAPHY

Garrey, W. E., 1916. *Am. Jour. Physiol.*, **39**: 313.
Lillie, R. S., 1906. *Am. Jour. Physiol.*, **17**: 89.
Loeb, J., 1900. *Am. Jour. Physiol.*, **3**: 327.
Osterhout, W. J. V., 1922. Injury, Recovery and Death, in Relation to Conductivity and Permeability. Philadelphia.

SPECIFIC INFLUENCE OF THE HOST ON THE LIGHT RESPONSES OF PARASITIC WATER MITES

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The common fresh-water mite *Unionicola ypsilophorus* var. *haldemani* (Piers), which lives as a parasite between the gills of the mussel *Anodonta cataracta* Say, exhibits interesting modifications in its behavior to light associated with its parasitic life. Mites which are removed from the influence of material from the host show a positive response to light, but when a small amount of water from the mantle cavity or an extract of gills of the host is added to water containing the mites there is an immediate and striking reversal to a negative state. A negative response to light is necessary to keep the parasites within the host and it was suggested (Welsh, 1930) that this reversal in light response might be considered adaptive and secondarily acquired. An attempt was made to determine the nature of the substance which causes the reversal and it was concluded that certain proteins or decomposition products of proteins were responsible for the reversal, which perhaps is something of the nature of a conditioned response with olfactory or taste organs being involved in the conditioning. In order to test this idea, in part, it was necessary to determine whether or not material from the host was specific in causing the reversal in the light responses of the mite. The present paper is concerned with the results of this investigation.

The majority of tests were made on the mites from *Anodonta*. although two other species from other fresh-water mussels were also tested. The experiment was simple and consisted merely in comparing the effect of water from the mantle cavity or a water extract of the gills of several different fresh-water mussels on the behavior to light of mites from a given host. Tests on the mites from *Anodonta* were made with material from the following fresh-water bivalves.

Lampsilis radiata (Gmelin)	Houghton's Pond Blue Hill, Mass.
Elliptio complanatus (Dillwyn)	
Sphaerium sulcatum	Belmont, Mass.

Cyclonais tuberculata (Raf.)
Eurynia iris (Lea)
Ligumia fasciola (Raf.)
Elliptio dilatatus (Raf.)
} Huron River, Delhi, Mich.

The tests were made under constant conditions of temperature and intensity of illumination, as it was found that both of these factors influenced the behavior of the mites. In a typical experiment six mites were placed in each of two small rectangular glass dishes 8 cm. long, 2 cm. wide, and 2 cm. high. The mites had previously been washed in several changes of water in order to remove any host material and had been free from the influence of the host for at least a day. The small jars containing the mites were placed in a large water bath where the temperature was maintained at approximately 18° C. The source of illumination was a 6 volt, 18 ampere ribbon filament lamp at a distance of thirty centimeters from the end of the tank. A glass window permitted the light to enter the tank, where it passed through twenty centimeters of water before it reached the jars containing the mites. The mites were always kept in 5 cc. of water and the gill extract prepared by grinding the gills in distilled water and then filtering, or the water from the mantle cavity, was added in 1 cc. quantities at the end of the jar towards the light. Whenever a reversal occurred the mites moved out of the region of extract faster than the diffusion took place.

Following is the record of a typical experiment:

April 22. Placed six mites in each of two jars *A* and *B*. All positive to light.

3:40. Added 1 cc. of extract of gills of *Cyclonais* to *A*. One mite stimulated in some way, and temporarily indifferent to light. returned in 30 seconds to light end of jar.

3:41. Added 1 cc. of extract of gills of *Anodonta* to jar *B*. Five mites immediately negative, one mite unaffected temporarily by extract.

3:45. All mites in *A* positive. All mites in *B* negative.

4:00. No change.

4:30. Removed and washed both lots of mites and returned to same jars in fresh water.

4:35. Added 1 cc. of extract of *Anodonta* gills to jar *A*. All mites immediately and actively negative.

4:40. Added 1 cc. of extract of *Cyclonais* gills to jar *B*. No indication of a response, all mites remaining positive.

This experiment was typical of all the tests made on the mites from *Anodonta* with material from each of the seven other species of mussels used. Gill extract or water from the mantle cavity of *Anodonta* only, was effective in bringing about a reversal in the light response of *Unionicola* from *Anodonta.*

Two other species of *Unionicola* were found, *Unionicola fossulata* (Koen) from *Cyclonais tuberculata,* and an undetermined species from *Lampsilis radiata.* Both species of mites were tested with material from their hosts as compared with extract from *Anodonta.* They both showed negative reactions to the same light intensity used in the tests on the mites from *Anodonta* but were found to be positive to a low light intensity obtained by using a neutral tint filter transmitting 0.1 per cent of the light used in the previous tests. A reversal in their light response could be brought about by material from their own host but not by material from *Anodonta.*

These results indicate that the material present in the host which causes a reversal in the light responses of parasitic water mites is specific for a particular host-parasite combination. It is possible that certain water mites have more than one host, as do certain parasitic copepods, in which case one would not expect to find the same specific influence of host on parasite. However, the majority of parasites are always found associated with a particular species as host and the results of these tests help to explain why this is true. A long continued parasitic or commensal life tends to modify an animal structurally and at the same time to modify the behavior of the animal and, as was pointed out in an earlier paper (Welsh, 1930), these mites which are found in *Anodonta* were probably primitively positive to light and the negative response has been acquired only after a long period of life within a particular species of mussel. The constant influence of some material from the host which stimulates the mites either through their olfactory or gustatory organs keeps them in a negative state as regards their behavior to light of a given intensity, but their removal from this influence causes a reversion to the primitive state.

The evidence thus far indicates a specific response on the part of fresh-water mites to some material from their host, and it is expected that similar associations exist in other host-parasite combinations. A study of these associations should reveal interesting modifications in behavior and yield further information regarding the intimate physiological relationships existing between closely associated animals.

LITERATURE CITED

Welsh, J. H., 1930. Reversal of Phototropism in a Parasitic Water Mite. *Biol. Bull.*, **59**: 165.

IS OSMOTIC HEMOLYSIS AN ALL-OR-NONE PHENOMENON?

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I

There are at present two opposed views concerning the relation between the disappearance of the erythrocyte and the amount of hemoglobin which it liberates during osmotic hemolysis. Some investigators (Ruzynyák, S., 1911; von Lieberman and von Fenyvessy, 1912; and J. Baron, 1928) believe that the disappearance is a gradual process resulting from a slow escape of hemoglobin, and hence that swollen though visible cells may have lost considerable quantities of this substance. Baron, for example, has reported that hemolysis induced by hypotonic salt solution may lead, as in one experiment which he cites, to the disappearance of 17 per cent of the cells while at the same time the amount of hemoglobin recovered in the surrounding solution is as high as 42 per cent. The conclusion is drawn that even visible cells must have lost a part of their hemoglobin. Baron and others have designated hemolytic processes of this type as " partial."

Another group of investigators (Dienes, L., 1911; H. Handovsky, 1912; S. C. Brooks, 1918; and G. Saslow, 1928–29) hold that the erythrocyte disappears with great rapidity at the time of hemolysis. Brooks, who worked chiefly with hemolysis by ultra-violet radiation, has summarized this concept in the following way: " When hemoglobin finally begins to diffuse from a given erythrocyte, the process is so quickly completed that it may ordinarily be regarded as instantaneous." According to this view the process is of the type customarily termed " all-or-none." A parallel situation is believed by many workers to be found in osmotic hemolysis. It is held by such workers that the cell subjected to osmotic changes which result in swelling, does not begin to lose its hemoglobin until it has attained a fairly definite volume, termed the " hemolytic volume," at which time a rapid outward diffusion of hemoglobin occurs to a point of equilibrium with the surrounding fluid.

The apparent " hemolytic volume " is believed to differ for different erythrocytes, even in blood from the same individual, hence the well-known range of resistance always encountered in experiments on osmotic hemolysis. In general, according to the second view that hemolysis is an all-or-none process, it ought to be possible to find a solution of such a concentration as to cause, in a given sample of blood, a complete loss of hemoglobin from all the cells whose resistance falls below a certain value, without any loss from the cells of higher resistance. According to the first view, however, that partial hemolysis is a phenomenon of common occurrence, such a sharp separation should not be possible.

The difference between these two views is of more than theoretical interest. If the first one is correct then little significance can be attached to the term " percentage hemolysis," and standards such as those used, for example, by Jacobs (1930), while reproducible and therefore of practical value, correspond to nothing encountered in actual experiments. If, on the other hand, hemolysis is actually an all-or-none phenomenon, then such standards represent not merely the apparent but also the true percentage of hemolysis, with a consequent gain in the significance of the results obtained.

The study of the relation between the disappearance of the cell and the liberation of hemoglobin involves measurements of two sorts: first, a count of the number of cells in the sample of blood employed, and second, a determination of the total hemoglobin content of the cells followed by an estimation after hemolysis has occurred of the number of cells undestroyed and of the hemoglobin content either of these cells or of the supernatant fluid, or preferably of both. An obvious point, which has been neglected by previous workers in securing ideal conditions for the outward diffusion of hemoglobin, is the use of a large volume of surrounding solution relative to the total volume of the cells, so that a true diffusion equilibrium would permit the escape of almost all of the hemoglobin that is free to leave the affected cells.

Under conditions where the outward passage of hemoglobin is not limited by an insufficient external volume, a comparison of the number of cells destroyed and the amount of hemoglobin liberated might conceivably lead to any one of three following results: (1) The amount of hemoglobin in the surrounding solution corresponds exactly to that contained in the cells that have disappeared. The process is therefore " all-or-none " in character. (2) The amount of hemoglobin in the surrounding solution is greater than that contained in the cells that have disappeared. This would suggest a " partial " process, at least in the case of some of the cells. (3) The amount of hemoglobin in the surrounding fluid is less than that contained in the cells that have disappeared. Such a condition would indicate a slight retention of hemoglobin by invisible cells. All of these possibilities are illustrated in Table I by data selected from the previous literature or obtained in the course of this investigation.

Strictly speaking, the "retention" type must always be present to some extent when hemolysis occurs in finite volumes of solution since a diffusion process can do no more than bring about an equality of distribution of hemoglobin between the cells and their surroundings. In previous work, where the whole blood was introduced into the hemolytic solution in the volume ratios of 1 to 1 to 1 to 5 (Baron, J., 1928; G. Saslow, 1928–29) this retention must of necessity have been great. In the present work, however, this effect has been minimized by employing a ratio of 1 to 2,000, thus providing an opportunity for an almost complete outward diffusion of the hemoglobin.

TABLE I

Summary of the Possible Types of Osmotic Hemolysis

Type	Cells Disappeared	Hemoglobin Appeared	Hemolysis in	Observer	Maximum Error of Method
	per cent	*per cent*			*per cent*
(1)	50	51	Dilute plasma	Saslow	7
"all-or-none"	75	76	NaCl, glycerol, ethylene glycol	author	4
(2) "partial"	17	42	Dilute plasma	Baron	?
(3) retention	66	58	Glycerol (early stages)	author	4

It should be noted further that a process of type (3) might be expected to occur as a temporary stage in the attainment of end results of type (1). Whether or not it would be observed would depend on the rapidity of the process and upon the conditions governing the visibility of the cells. Theoretically also a combination of types (2) and (3) might conceivably at times simulate type (1). That such a combination of effects would occur so exactly and consistently in an extended series of experiments as to lead to erroneous conclusions is, however, extremely unlikely.

In the present investigation it has been found that the condition described as type (1) is always present when the hemolytic system has attained a final equilibrium. In certain cases, for example, during the course of hemolysis produced by glycerol solutions, a temporary retention of some hemoglobin is exhibited during the early stages of the process; but at final equilibrium the relation is the same as before, namely, "all-or-none." No evidence whatever has been found under the conditions of these experiments of the escape of hemoglobin from visible cells; that is, of "partial hemolysis" of individual erythrocytes.

II

To determine accurately the relation between the number of erythrocytes that disappear and the amount of hemoglobin liberated in a given hemolytic system it is necessary that a great number of careful cell counts be made. In the present work these counts were made with the usual counting chamber, the areas customarily used for white cells being employed, and at least 10 of these areas of 1.0 sq. mm. each being counted. This procedure involved a count of from 1,500 to 2,000 cells for each solution. By counting a large number of unit areas (160) and also a large number of cells the accuracy of the cell count was increased so that the maximum error was 2 per cent as determined by the method of Student (1907). The use of dilute cell suspensions (1 to 2,000) eliminated the difficulties encountered by previous workers owing to the presence of so-called "ghost" cells. This point will be considered in detail later, and has been touched upon here solely to stress the fact that at no time during the course of these experiments did the difficulty which previous investigators (Baron; Saslow) found in distinguishing between "stromata" and intact cells arise.

The method of hemoglobin estimation employed in all of these experiments was a new one particularly well adapted to this purpose. It was worked out in conjunction with Dr. W. R. Amberson and Dr. D. R. Stewart, and will be described in detail elsewhere (1931). The principle involved in the method is that of the optical pyrometer, and its most valuable features are the high sensitivity and accuracy that can be obtained. The sensitivity proved to be entirely adequate to deal with the minute amounts of hemoglobin that the use of very dilute cell suspensions necessitated. Thus, in experiments in which whole blood was mixed with distilled water in the proportion of 1 to 2,000 it was found that between this concentration and zero concentration of hemoglobin a series of at least one hundred readings could be obtained by the pyrometer. This represents a maximum error in the hemoglobin readings even at these unusually low concentrations of 1 per cent. The term "percentage of hemoglobin" is everywhere used in this paper to designate the amount of hemoglobin in the surrounding fluid of a given hemolytic solution relative to that contained in the total number of cells employed.

It is known that whole blood on standing even for a short period of time (2 to 3 hours) and at a low temperature (3° to 5° C.) often undergoes a slight degree of hemolysis which may range between 0 and 4 per cent. The amount of hemoglobin in the plasma introduced with the whole blood into the hemolytic solutions, as well as other pigments that may be present, must, therefore, be corrected for in the hemoglobin determinations. This has been accomplished by diluting whole blood in the proportions of 1 to 2,000 in isotonic saline and then rapidly (10 minutes) centrifuging the cells out. A pyrometer reading on the supernatant fluid gives the error due to the hemoglobin and other pigments in the plasma. This correction for plasma error has been applied in all the experiments here recorded.

The dilution steps involved in the hemoglobin determination, the preparation of the cell suspensions and the cell counts were found to have an error of not more than 1 per cent. Since the cell counts involved an error of approximately 2 per cent and the hemoglobin determinations of not more than 1 per cent, the total maximum error in comparison of the disappearance of cells and of the appearance of hemoglobin in the solution was of the order of magnitude of 4 per cent. It will be noted that in Table II and in Figs. 1 and 2 the agreement is well within this figure.

TABLE II

Data Illustrative of the Accuracy of the Method

A. Percentage of hemoglobin appeared	3	3	12	20	25	30	51	62	77	95
B. Percentage of hemoglobin in cells remaining	99	96	89	78	78	70	50	39	21	4
A + B	102	99	101	98	103	100	101	101	98	99

Table II also gives another very important check on the accuracy of the method in demonstrating that the hemoglobin which was recoverable from the visible cells plus the hemoglobin in the supernatant fluid is equivalent, within the experimental error, to that contained in the original number of cells. There is no evidence that this very necessary test has been made by previous workers and it is apparently to its omission that much of the confusion in the literature is attributable.

A point of particular importance in obtaining the results here recorded is strict attention to the influence of the factors discussed by Jacobs and Parpart (1931), of which temperature, pH and the attainment of a final equilibrium are the most important. A recent worker (Saslow, G., 1928–29) states that "Most of the experiments performed were failures because of the difficulties above enumerated: lack of control of degree of hemolysis and unsatisfactoriness of the cell count."

Only in three instances was he able to secure a suitable degree of hemolysis, and this unfortunately fell within the narrow range of from 40 to 60 per cent. In the present work, by a careful control of the factors influencing the degree of hemolysis, namely temperature, pH and the attainment of equilibrium, and by the use of dilute cell suspensions, thus allowing a practically complete outward diffusion of hemoglobin from the affected cells which removes the necessity for determining " ghosts," it has been found possible to obtain very readily any desired degree of hemolysis. This may be observed in Fig. 1, where the entire range from zero to 100 per cent has been covered with gaps of no more than 5 per cent.

III

Osmotic hemolysis has been used throughout this work. The experiments performed fall into two classes. Those belonging to the first class involved the use of a non-penetrating substance, sodium chloride, in hypotonic solutions of concentrations so chosen as to bring about a disappearance of some but not all of the erythrocytes present in the suspension. By varying the concentration in small steps it was possible to cover the entire range from zero to 100 per cent hemolysis. After the hemolytic system had reached its final equilibrium condition or after hemolysis had been checked in the manner described below, a comparison was made between the number of cells that had disappeared and the amount of hemoglobin that had been liberated. In the second group of experiments hemolysis was allowed to occur in solutions, originally isosmotic with blood, of the penetrating substance glycerol and, in a few cases, ethylene glycol. The hemolysis produced by the penetrating substances was checked at various points by the addition of sodium chloride in the proper amount and comparisons were made as before between the liberation of hemoglobin and the disappearance of cells. Because of their greater simplicity, the experiments involving solely the entrance of water into the cells will be described first, and those involving the penetration of the solute as well will be dealt with in the following section.

As has already been mentioned, the factors of temperature and pH must be so regulated that their effect on the degree of hemolysis attained is a constant one. The temperature of the hemolytic solutions employed in these experiments was maintained at 20° ± 0.1° C. by means of a water bath. The pH of the hypotonic solutions was controlled by the addition of a small amount of phosphate buffer. All solutions were prepared from a stock solution consisting of 14 parts of molar NaCl and 1 part of molar Na_2HPO_4, brought to a pH of 7.0 by the addition of a trace of concentrated HCl. Upon dilution of this stock solution to concentrations between 0.5M and 0.05M, the pH of the resulting solutions was 7.40 within the limits of accuracy of the quinhydrone electrode (± 0.02). Since the pH of the blood used is originally not far from this point, the comparatively slight buffering of the solution is sufficient for all practical purposes.

In the experiments involving the simplest type of osmotic hemolysis the procedure was as follows: To 50 cc. amounts of various hypotonic salt solutions, usually differing from one another by 0.001M, 25 cu. mm. of whole blood, defibrinated by whipping, was added from a calibrated hemoglobin pipette after the solutions had been brought to a temperature of 20° C. ± 0.1°, in a water bath. Following the addition of blood they were gently and continuously stirred, at the temperature stated, for a period of one hour, which is more than sufficient for the attainment of equilibrium (Jacobs and Parpart, 1931). A small portion of the solution was then removed for the cell count, while the rest was centrifuged at 2,000 r.p.m. for 15 minutes and the supernatant fluid siphoned off for the hemoglobin estimation. The time allowed for centrifuging was shown to be sufficient by two procedures, namely, by microscopic examination of the supernatant fluid which revealed the presence of no cells, and by measurement with the optical pyrometer which gave the same transmission values for the fluid whether it had been centrifuged for 10 minutes or for one hour.

TABLE III

Data on the Blood of One Animal (Ox) Illustrating the Applicability of the All-or-none Concept at Equilibrium

Concentration of NaCl + Na_2HPO_4	A. Cells Disappeared	B. Hemoglobin Appeared*	B–A
M/l	*per cent*	*per cent*	
0.154	0	0	—
0.115	3	3	0
0.110	9	12	+3
0.100	22	23	+1
0.095	27	30	+3
0.090	64	62	−2
0.085	79	77	−2
0.080	92	95	+3

* After correction for the plasma error.

Table III presents the results obtained in a typical experiment of this sort with ox blood. The concentrations indicated represent dilutions of the original stock solution containing both NaCl and Na_2HPO_4 whose concentration was somewhat arbitrarily taken as unity. Though osmotically the concentrations given are not exactly equivalent to similar ones of pure NaCl, the differences are very small and the solutions are entirely reproducible. The values given in column A represent the difference between the total number of cells employed and the number of cells remaining when the hemolytic system was at equilibrium, and are expressed on a percentage basis. Column B gives the percentage of hemoglobin in the surrounding solution, which was determined after correction for plasma error as previously described.

Inspection of Table III shows that the hemoglobin which appears in the supernatant solution corresponds, within the limits of experimental error, almost exactly with that originally contained in the cells that have disappeared. There is no evidence, therefore, at the end of the time in question (1 hour) of any appreciable retention of hemoglobin nor of partial hemolysis of cells still visible. The process under these conditions appears to be strictly " all-or-none " in character.

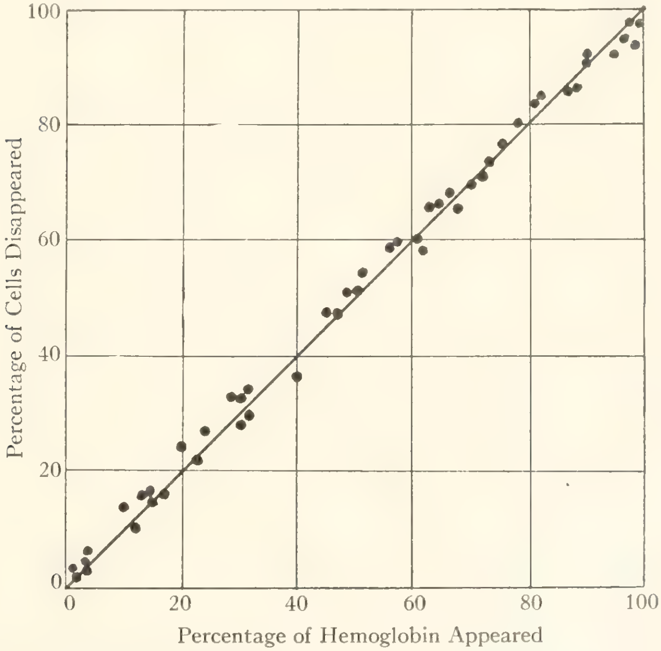

FIG. 1. The liberation of hemoglobin from ox erythrocytes in hypotonic NaCl solutions under equilibrium conditions.

More extensive corroborative data are shown in Fig. 1, where the results of 50 experiments in which the blood of 12 animals was used are plotted. The solid diagonal line represents the condition that should obtain if the hemolytic process, at equilibrium, is all-or-none in character. The experimental points scatter about this line within the error of the method; that is, with a maximum deviation of 4 per cent and an average deviation of 1.7 per cent. Since it is inconceivable that in such a large number of experiments as exact an agreement as this could be obtained by a fortuitous combination of retention of hemoglobin and of partial hemolysis, the conclusion seems inescapable that an all-or-none process is being dealt with.

The results and conclusions summarized in Tables II and III and in Fig. 1 were obtained with ox blood, while the conflicting data of Baron (1928) and Saslow (1928-29) were based upon experiments on human blood. Since there is a marked difference in the osmotic resistance of human blood and of ox blood it seemed advisable to perform a number of similar experiments on the former type of blood. These experiments are represented in Fig. 2, in which the diagonal line has the same significance as before. It will be noted from this figure that the evidence of an all-or-none relationship is as unmistakable in the case of human as in that of ox blood.

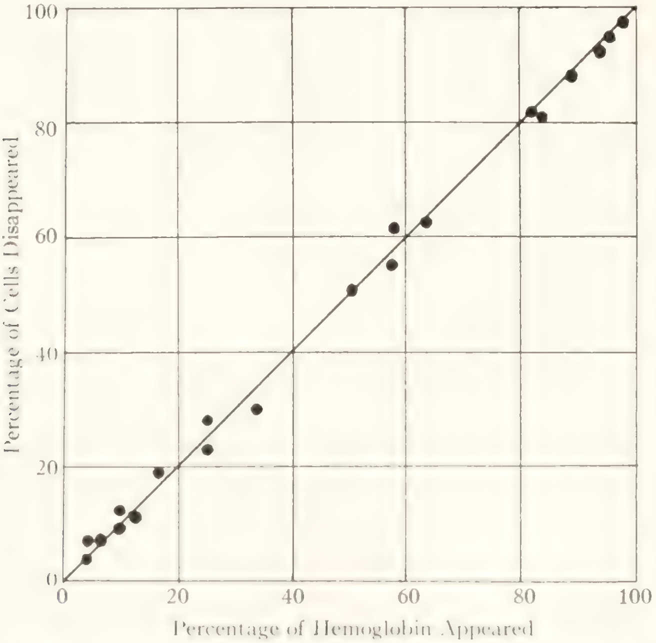

FIG. 2. The liberation of hemoglobin from human erythrocytes in hypotonic NaCl under equilibrium conditions.

In all of the experiments so far described hemolysis has been allowed to go to complete equilibrium. The question arises whether there is a similar agreement between the hemoglobin that has escaped and the number of cells that have disappeared at times before the final equilibrium of the system has been attained. Stated in a more concrete manner, if in a given hemolytic system the concentration is such that the final equilibrium will involve the destruction of, for example, 75 per cent of the cells present, will the all-or-none concept hold when only 25 per cent of the cells have undergone hemolysis?

To answer this question, the rate of hemolysis of ox blood in hypotonic salt solutions in which at equilibrium there remained some percentage of the cells unhemolyzed, was first followed by the method of Jacobs (1930), and in this way it was determined at what time after setting up a given experiment any desired degree of apparent hemolysis had been attained. It was then possible to repeat the experiment and to stop the hemolysis at the chosen point and to make comparisons as before. In a number of experiments of this type 25 cu. mm. of whole blood of the ox was added to 25 cc. amounts of hypotonic saline solutions whose hemolysis-time curves had been determined as above. At the end of a definite time (15 seconds to 2 minutes) 25 cc. of salt solution of a concentration to make the whole isotonic (0.154M) was suddenly mixed with the hemolyzing solution, thus stopping hemolysis. As before, the temperature was kept at 20° C. and the pH at 7.4. Cell counts were then made on a portion of the solution in which hemolysis had been stopped, while the remainder was centrifuged and the supernatant fluid used for the hemoglobin estimations in the usual manner. A series of these determinations led to the results recorded in Table IV.

It is evident from this table that the agreement between the percentage of the cells that have disappeared and the hemoglobin that has been liberated is as good as it was in the cases where the final equilibrium had been reached. The all-or-none concept, therefore, is not limited merely to the end-stage of osmotic hemolysis of this type but probably applies throughout the entire process. The conclusion would seem to be warranted that in studies on the kinetics of osmotic hemolysis the cell may be assumed to liberate all of its hemoglobin at the time of its disappearance.

IV

Osmotic hemolysis in solutions of penetrating substances is a somewhat more complicated process than that so far described since the rate of entrance into the cell of the solute as well as that of water is involved. Since previous workers have apparently not studied the nature of the hemolytic processes induced by these substances, it appeared of interest to determine whether they might also be associated with an all-or-none type of hemolysis. The substance chiefly studied, namely glycerol, was chosen not only because it is relatively non-toxic, but because its rate of penetration into the erythrocyte of the ox is sufficiently slow so that the hemolytic process can be stopped at any desired point by the addition of salt in proper concentration. A typical experiment may now be described.

TABLE IV

Effect of Stopping Hemolysis Before the Attainment of Equilibrium

Concentration of NaCl + Na_2HPO_4	A. Cells Disappeared	B. Hemoglobin Appeared	B–A
M/l	*per cent*	*per cent*	
0.090	71	72	+1
0.090	31	27	−4
0.090	36	35	−1
0.090	37	38	+1
0.088	75	74	−1
0.088	35	32	−3
0.088	45	43	−2
0.088	49	52	+3
0.085	67	64	−3
0.085	20	22	+2
0.085	33	29	−4
0.085	40	40	0
0.082	80	78	−2
0.082	55	53	−2
0.082	57	58	+1
0.082	61	58	−3

Whole defibrinated blood of an ox was introduced into an isosmotic solution of glycerol, in the proportion of 25 cu. mm. of blood to 25 cc. of 0.3M glycerol. The solution was gently stirred and kept at a temperature of 20° C. and the rate at which hemolysis proceeded determined by the method of Jacobs (1930). Until about 35 minutes after setting up such a system, no hemolysis was found to occur; between 35 and 55 minutes the process proceeded fairly rapidly from 0 to 100 per cent. It was then a simple matter in subsequent experiments to stop the hemolysis at any desired point between these two time intervals by the addition to 25 cc. of the suspension undergoing hemolysis of 25 cc. of a solution at pH 7.4 containing 0.308M NaCl + Na_2HPO_4 and 0.3M glycerol. After the addition of this solution the whole was equilibrated at 20° C. with stirring for a period of one hour, at which time cell counts and estimations of the hemoglobin liberated were made in the manner described in the previous section.

Blood samples from five animals were tested in this manner and the results are summarized in Fig. 3. In this figure, as in the two previous ones, the diagonal line represents the theoretical result that should obtain if the process is all-or-none. It will be observed that in this case the experimental points in these determinations scatter on one side of this line in the direction of an hemolytic process exhibiting a retention of hemoglobin. The deviations, which amount to 5 to 12 per cent, are too great to be accounted for by experimental errors alone.

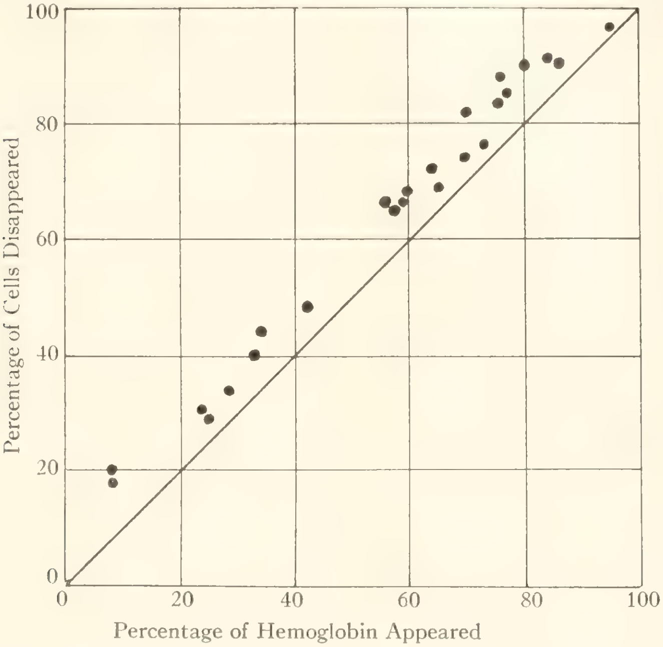

FIG. 3. The liberation of hemoglobin from ox erythrocytes during the early stages of hemolysis by glycerol.

Two possible explanations of these results suggest themselves. The first is that the penetration of the glycerol might so alter the refractive index of a number of the more swollen cells that they do not appear in the count though they still retain all or a part of their hemoglobin. The second is that the hemolytic system may not have attained its final equilibrium at the time the observations were made.

As a test of the former possibility the following experiment was performed. Whole blood of the ox was pipetted into a hypotonic saline solution that would induce a slight degree of hemolysis, in the proportion of 25 cu. mm. of blood in 50 cc. of salt solution at pH 7.4. These solutions were equilibrated for one hour with gentle stirring, at 20° C.

At the end of the equilibration period 25 cc. was removed for cell count and hemoglobin estimation. To the remaining portion was added an equal volume of a solution containing the same concentration of salt plus 0.6M glycerol. Thus the salt concentration remained unchanged, while the solution became isosmotic with respect to glycerol. This latter solution was equilibrated for one hour in the same way, at which time cell counts and hemoglobin determinations were again performed. Due allowance was made for the one-half dilution necessitated by the procedure. The results of several such experiments are presented in Table V.

Table V

Effect of Glycerol on the Refractive Index of the Erythrocyte

Before the Addition of Glycerol			After the Addition of Glycerol		
A. Cells Disappeared	B. Hemoglobin Appeared	B-A	A. Cells Disappeared	B. Hemoglobin Appeared	B-A
per cent	*per cent*		*per cent*	*per cent*	
35	32	−3	40	38	−2
31	29	−2	32	33	+1
28	29	+1	33	32	−1
50	51	+1	52	51	−1

Had the glycerol in any way affected the refractive index of the corpuscles, then the data obtained after the addition of glycerol should have departed from the all-or-none relationship by an amount comparable to that observed in Fig. 3. As no such shift was observed, it may be concluded that glycerol does not appreciably alter the refractive index of the erythrocytes. The explanation, therefore, of the discrepancy between the hemoglobin liberated and the cells disappeared in a hemolytic system of the glycerol type would appear to lie along the lines of the second suggestion, namely the time required for the attainment of equilibrium.

To test this point, whole blood of the ox was equilibrated for one and for four hours, respectively, in hypotonic saline solutions containing varying concentrations of glycerol between 0.03M and 0.3M. As before, 25 cu. mm. of blood was added to 50 cc. of each solution. To make the experimental conditions comparable, half of the solution was removed for the determinations at the end of one hour, the rest being equilibrated in the customary way for an additional period of three hours. The concentrations of salt and of glycerol used are shown in Table VI.

Examination of this table shows that in the absence of glycerol, as in previous experiments, the hemoglobin liberated at the end of either one or four hours agrees within the experimental error with that in the cells which have disappeared. In the presence of glycerol, however, at the end of one hour there is an apparent retention of hemoglobin similar to that indicated in Fig. 3, whereas this had completely disappeared by the end of four hours. In brief, it would appear that at the final equilibrium the conditions are the same as before, but that the final equilibrium is longer in being attained.

TABLE VI

Production by Glycerol of an All-or-none Type of Hemolysis, at Equilibrium

Solution	After 1 Hour			After 4 Hours		
	A. Percentage of Cells Disappeared	B. Percentage of Hemoglobin Appeared	B–A	A. Percentage of Cells Disappeared	B. Percentage of Hemoglobin Appeared	B–A
0.090 M NaCl + Na_2HPO_4	90	88	−2	89	88	−1
	90	90	0	88	89	+1
Same + 0.03 M glycerol	68	60	−8	88	87	−1
Same + 0.10 M glycerol	66	58	−8	93	94	+1
Same + 0.30 M glycerol	34	28	−6	91	92	+1

As to the cause of this delay in the escape of hemoglobin, several possibilities might be suggested. One of the most plausible is that the retention of hemoglobin during the first part of the hemolytic process may be due to a blocking of the hypothetical " pores " by which it is frequently assumed to leave the swollen cells, either because of the relatively large size of the glycerol molecule or because of its adsorption on the walls of the pores. In either case, the effect of the glycerol would be to decrease the surface area through which hemoglobin may leave the cell. The data in Table VI lend confirmation to this view, for not only does the presence of a very small amount of glycerol (0.03M) markedly slow the rate of disappearance of the cells, but it also causes marked temporary retention of hemoglobin. This is true, in spite of the fact that the solution of 0.03M glycerol and of 0.09M NaCl is osmotically equivalent to a 0.105M solution of NaCl, a concentration which of itself rapidly produced about 15 per cent hemolysis. It may further be determined from this table that the initial distance of the hemolytic system from osmotic equilibrium does not influence the amount of retention. As has already been pointed out, the extent of this retention is outside the limits of experimental error of the methods here employed. It averages about 8 per cent as compared with a maximum experimental error of 4 per cent and, as will be observed in Fig. 3, the deviation is consistently in the same direction.

As contrasted with glycerol, ethylene glycol, a closely related substance of lower molecular weight, fails to produce any observable retention of hemoglobin even during the early stages of the hemolytic process. Its behavior is indicated by the data given in Table VII, which were secured by equilibrating for one hour the usual dilution of ox blood in 0.3M ethylene glycol made up in the salt solutions of the concentration indicated in the table. It will be noted that the presence of the ethylene glycol affects neither the rapid attainment of equilibrium nor the all-or-none character of the hemolysis that the hypotonic solutions alone would have exhibited. Except for the lower molecular weight and molecular volume there is no obvious reason why this substance should differ so markedly from glycerol.

Table VII

Hemolysis by Ethylene Glycol

Concentration of $NaCl + Na_2HPO_4$ in 0.3 M Ethylene Glycol	A. Cells Disappeared	B. Hemoglobin Appeared	B–A
	per cent	*per cent*	
0.085	15	19	+4
0.085	19	20	+1
0.080	45	47	+2
0.080	46	46	0
0.078	60	58	−2
0.078	60	62	+2
0.075	75	76	+1
0.075	76	76	0

V

The results that have been obtained fail entirely to confirm Baron's (1929) contention that osmotic hemolysis is a "partial" process. Hemolysis of this type has constantly been found to be an all-or-none phenomenon both under equilibrium conditions and while in progress, except for the temporary retention of hemoglobin that occurs during the early stages produced by a penetrating substance like glycerol.

Even in this case, however, the end-point finally attained by the system is the same as in that found under the simpler conditions where the entrance of water alone is involved.

It was mentioned previously that Baron's results were secured by the use of 1:1 to 1:6 dilutions of whole blood with water. To make cell counts and hemoglobin estimations he added sufficient hypertonic salt solution to make the dilute hemolytic solution isotonic. After obtaining cell counts he then centrifuged the solution and made the hemoglobin estimation on the supernatant fluid. The futility of such a procedure might, however, have been recognized from the results of Bayliss (1924–25), or of Adair, Barcroft and Bock (1921) and others. These workers have clearly demonstrated that when whole blood is diluted with water in proportions such as those mentioned above, the corpuscles swell and lose hemoglobin only to a degree that corresponds with a diffusion equilibrium between the cells and the external solution. If at this point salt is added to make the solution isotonic, the cells shrink, thus trapping in them sufficient hemoglobin so that they again become visible. Bayliss (1924–25) discussed these conditions as follows: " Suppose the ghosts had a volume two and one-half times the volume of the original corpuscles and contained the same concentration of hemoglobin as the surrounding fluid. Then, if on shrinking to their normal size they become more or less impermeable to hemoglobin, they might contain finally a hemoglobin concentration some two to three times that of the external fluid."

It would appear, therefore, that Baron, when making his cell counts, must have included a number of cells whose visibility had been restored after they had previously lost a portion of their hemoglobin. That the hemoglobin was not more completely lost was obviously due to the small volume of the external solution. Under these experimental conditions it is not surprising that no exact correspondence could be obtained between the number of cells that had disappeared and the amount of hemoglobin in the surrounding medium. As has been mentioned, Saslow (1928–29), in his studies on hypotonic saline hemolysis, using the method of Baron, obtained data which contradicted those of the latter author. The reason for this discrepancy is perhaps furnished by Saslow's statement that before making the cell count he pipetted off and discarded the " stromata." The so-called " stromata " were probably cells which had lost their hemoglobin to equilibrium with the surrounding fluid and by discarding them he made his cell count on cells which had not lost hemoglobin, and naturally the cell count corresponded with the hemoglobin in those cells. If he had determined not only the amount of hemoglobin in the cells undestroyed but also that in the surrounding fluid it is likely that the two amounts would have totalled considerably less than 100 per cent. However, this crucial test was not made.

An attempt has been made, by the use of Saslow's dilution of blood with distilled water, to determine the hemoglobin content of the supernatant fluid after " reversion " of the cells by means of sufficient NaCl to make the solution isotonic followed by centrifuging at 5,000 r.p.m. for one and three hours. A clear supernatant fluid is obtained in this way, but further addition of salt to it causes it to become clouded, and microscopic examination shows this cloudiness to be due to the reappearance of cells. If this latter cloudy supernatant fluid is allowed to stand for some fifteen to thirty minutes it again becomes relatively clear and can be made cloudy again by making it still more hypertonic with salt. A short time later it again becomes clear. Thus, in the method of Saslow, and also in that of Baron, the " clear " supernatant fluid really must contain cells which have approximately the same specific gravity and, more important, the same hemoglobin content as the surrounding fluid. Because of this circumstance hemoglobin determinations on the supernatant liquid of very concentrated suspensions, whether made directly, as by Baron (1928), or indirectly, as by Saslow (1928–29), are unreliable.

Consideration of these facts leads to the conclusion that only when conditions are created such that the volume of the cells at the time of hemolysis is relatively small as compared with that of the surrounding fluid, can the nature of hemolysis be determined. Dilution of whole blood in hypotonic salt solution in the proportion of 1:2,000 adopted in these experiments leads to practically infinite dilution of the hemoglobin content of the hemolyzed cells. Under these conditions " ghost " cells and the phenomenon of " reversion " introduce no complications and it becomes possible to demonstrate that osmotic hemolysis is an all-or-none phenomenon, and that the term " percentage hemolysis " has a real significance.

SUMMARY

1. Hemolysis produced by hypotonic sodium chloride is of an all-or-none type, that is, hemoglobin either fails to escape from the erythrocyte or does so completely up to the point permitted by the attainment of a diffusion equilibrium in the system.

2. The same all-or-none character is observed when, instead of permitting the hemolytic process to proceed to its original equilibrium position, it is stopped at an intermediate point by the addition of sodium chloride.

3. In hemolysis in solutions of penetrating substances such as glycerol and ethylene glycol, the final equilibrium obtained after checking the process by the addition of sodium chloride likewise indicates an all-or-none relationship.

4. In glycerol solutions the liberation of hemoglobin lags somewhat behind the disappearance of the cells and the final equilibrium is rather slowly attained.

I am greatly indebted to Dr. M. H. Jacobs for the suggestion of this problem and for his helpful criticism.

BIBLIOGRAPHY

Adair, G. S., J. Barcroft, and A. V. Bock, 1921. *Jour. Physiol.,* **55**: 332.

Baron, J., 1928. *Pflüger's Arch.,* **220**: 242.

Bayliss, L. E., 1924. *Jour. Physiol.,* **59**: 48.

Brooks, S. C., 1918. *Jour. Gen. Physiol.,* **1**: 61–80.

Dienes, L., 1911. *Biochem. Zeitschr.,* **33**: 268–274.

Handovsky, H., 1912. *Arch. exper. Path. u. Pharm.,* **69**: 412.

Jacobs, M. H., 1930. *Biol. Bull.,* **58**: 104.

Jacobs, M. H., and A. K. Parpart, 1931. *Biol. Bull.,* **60**: 95.

Parpart, A. K., W. R. Amberson, and D. R. Stewart, 1931. *Biol. Bull.,* **61**: 518.

Rusznyák, S., 1911. *Biochem. Zeitschr.,* **36**: 394.

Saslow, G., 1928–29. *Quart. Jour. Exper. Physiol.,* **19**: 329.

Student, 1907. *Biometrika,* **5**: 351.

Von Liebermann, L., and B. von Fenyvessy, 1912. *Zeitschr. Immunitätsforsch. Orig.,* **12**: 417.

THE DETERMINATION OF HEMOGLOBIN CONCENTRATION IN DILUTE SOLUTIONS

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I

Colorimetric methods for hemoglobin determinations have been found to have an error which varies between 1 and 5 per cent (Schwentker, F. F., 1929). The chemical procedures for the quantitative estimation of hemoglobin, such as the measurement of the iron content (Fowweather, F. S., 1926) and the carbon monoxide capacity (Van Slyke, D. D., and A. Hiller, 1928), have a greater accuracy, *i.e.*, .5 to 1 per cent. These methods, however, necessitate the use of hemoglobin solutions of high concentration.

The investigator desiring to make a large number of hemoglobin determinations in a reasonable time, and under circumstances where only minute amounts of hemoglobin are available, must have recourse to some other method. An optical system suggests itself, but it should combine simplicity of operation with a high degree of accuracy.

The hemoglobin determinations reported in the preceding paper (Parpart, 1931) were secured by a method which employs the principle of the optical pyrometer. The equipment necessary is readily procurable; the determinations may be made with great rapidity, and the intrinsic error of the method is 1 per cent. A striking feature of this optical system is its sensitivity for low hemoglobin concentrations. To determine the sensitivity we have studied a solution of hemoglobin, standardized by the iron content method, kindly furnished by Dr. W. C. Stadie, and have found that the apparatus used is capable of detecting changes in hemoglobin concentration of the order of 1.2×10^{-5} mM. (M. W. = 68,000) in the range from 1.3 mg. to 0 mg. per 50 cc. of solution. In most uses of the method absolute values are not determined, since accurate relative values are sufficient.

The apparatus should also prove useful in quantitative determinations on the related pigments in both animals and plants, especially under circumstances where these pigments are procurable only in small amounts.

II

Our application of the pyrometer principle to this problem employs an optical system in which the light intensity of a line filament in a pyrometer lamp is matched against a diffuse background of fixed intensity. Hemoglobin solutions of different concentrations are placed in a 20 cm. polariscope tube between the two light sources. The apparatus is similar to that described by Amberson (1922, I).

A lamp, termed the pyrometer lamp (*C*), is placed at a distance of about 1 foot from another lamp, the background lamp (*E*). A small telescope (*B*) fixed at its focal distance from the pyrometer lamp, makes it possible to view a portion of the filament of this lamp against a background of light emitted by the background lamp and rendered diffuse by a ground glass plate set in at one end of the polariscope tube. Details of this arrangement may be observed in Fig. 1.

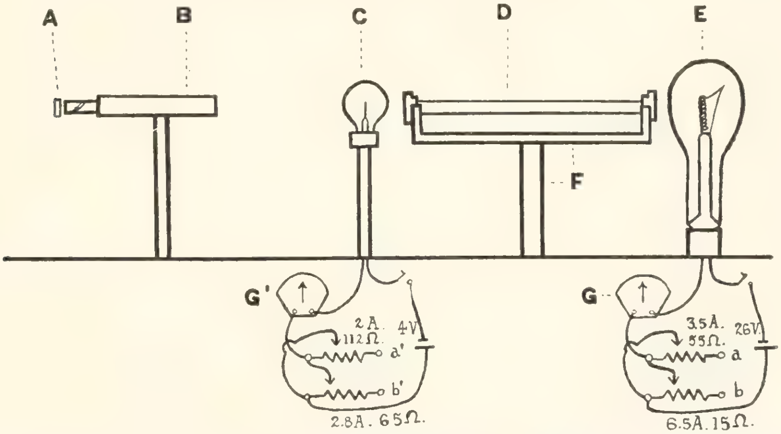

Fig. 1. Diagram of the optical pyrometer as applied to the estimation of hemoglobin concentration in dilute solutions. The symbols are explained in the text.

The pyrometer lamp is a small 3-volt bulb with a U-shaped filament of tungsten of about 0.2 mm. in diameter; while the background lamp is a Mazda bulb rated at 26 volts, 6.6 amperes, 2,500 L. Both lamps are run by storage batteries since line circuits fluctuate too much to permit adequate control. Frequent observations are made of an ammeter (*G*) in series with the background lamp circuit and its intensity is maintained constant by adjustment of a coarse-fine parallel resistance (*a* and *b*), in series. The current passing through the pyrometer lamp is recorded by a milliammeter (*G′*), and can be varied in steps of about 0.25 milliampere by a coarse-fine parallel resistance (*a′* and *b′*) in series.

In these hemoglobin determinations it is not necessary to know the relation existing between the intensity of the pyrometer lamp and the current passing through it, since all readings are evaluated by reference to a calibrated curve made with hemoglobin solutions of known concentration.

An essential feature in securing high sensitivity is the use of a green glass filter (*A*) which is placed at the eyepiece of the telescope. The transmission characteristics of this filter presented in Fig. 2 were obtained spectrophotometrically through the kindness of Dr. D. L. Drabkin. It will be noticed that the filter has its maximum transmission in the region of maximum absorption by hemoglobin, whether the latter is in the oxygenated or reduced condition, or in the form of methemoglobin or acid hematin.

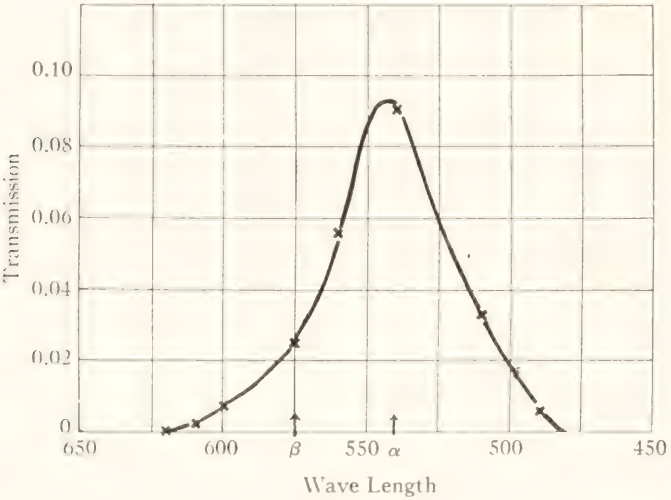

FIG. 2. Transmission curve for the green filter.

A 20 cm. polariscope tube (*D*), containing the hemoglobin solution, is placed on a rigid stand (*F*) between the two lamps. The usual plate-glass disc covers the end of the tube facing the pyrometer lamp, while at the other end there is a glass disc ground on one surface. This latter disc serves to diffuse the light from the background filament. Both of these discs must always be replaced in their original position as rotation will vary their transmission.

The readings have been taken with the background lamp set and stabilized at 4.7 amperes. With the hemoglobin solution in place, the amperage of the pyrometer filament is varied until the portion selected for observation just disappears, proceeding in every case from dark to light. The milliamperage of the pyrometer lamp is recorded and the readings made in triplicate. After a brief amount of practice these readings check within 0.5 to 1.0 milliamperes.

III

Due allowance must be made for certain variations in the optical system and for other possible sources of error, and these will now be considered. Slight changes in the brightness of the lamps and variations in transmission of the interposed glass surfaces have been observed from week to week. These changes have been determined by taking readings on distilled water before and after each experiment. Such readings taken as much as 24 hours apart have always checked within the limits of error of reading and hence the lamps and the transmission of the glass may be considered constant for this period.

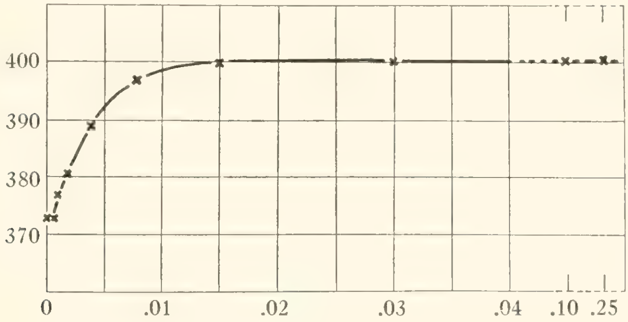

FIG. 3. Effect of salt concentration on the transmission of a dilute hemoglobin solution (dilution 1 to 4,000). Readings were taken 20 hours after preparation of the solution. Ordinates represent readings of milliammeter; abscissæ, concentration of NaCl in mols per liter.

When very dilute solutions of hemoglobin are employed, a few details must be carefully controlled. The dilute hemoglobin solutions are obtained by hemolyzing whole blood in distilled water in the proportion 1 to 2,000. At this dilution it can be shown that the absorption of light is influenced by some factor or factors in addition to hemoglobin concentration itself, since the addition of salt increases the transmission. Figure 3 shows the relation between salt concentration and transmission (measured in milliamperes). The reason for the decreased transmission between 0.015M salt and distilled water is uncertain; that the precipitation of serum globulins may play a part in it seems possible, but as their concentration is very small at such dilutions they probably do not account for the entire effect. This factor has been taken into account in all determinations by making the hemoglobin solutions up to

0.10M $NaCl + Na_2HPO_4$ (in the ratio 14 parts NaCl to 1 part Na_2HPO_4) at pH 7.40. All dilutions have been made with a salt solution of the same concentration.

Changes in hydrogen ion concentration have no effect upon the reading between pH 5.50 and 8.50 (determinations in steps of 0.2 pH units) nor does the presence of 1 per cent HCl alter the reading. In a like manner oxygenation or reduction has no effect. This might be anticipated from the transmission characteristics of the green filter used.

The plasma introduced with the whole blood in preparing the dilute solutions of hemoglobin constitutes a small but variable source of error. This error appears to be partly the result of a slight degree of hemolysis which takes place in the whole blood upon standing, though it is kept at about 2° C. until used. The error varies between 1 and 4 per cent, and as it is determinable, a correction can be applied. This factor, due to pigments in the plasma, may be determined by suspension of whole blood in isotonic salt solution in the same dilution as that used to prepare the hemoglobin solution. The cells are then removed by centrifuging. The supernatant fluid is read by the pyrometer and compared with the value for distilled water.

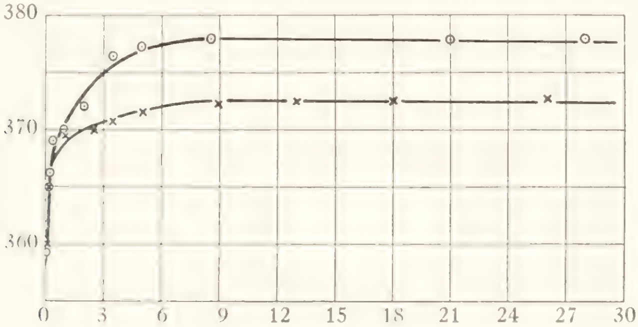

FIG. 4. Shift with time of the transmission of dilute hemoglobin solutions (dilution 1 to 2,000). Ordinates represent readings of milliammeter; abscissæ, time elapsed since the preparation of the hemoglobin solutions.

Another important factor influencing the determination in dilute solutions of hemoglobin is the time elapsed since the preparation of the solution. Readings were taken on samples of the same solution at a series of time intervals after its preparation. In Fig. 4 the results of two such experiments are plotted. Between the time of preparation of the solution and 4 to 8 hours later the transmission increases, which causes an apparent decrease in the hemoglobin concentration; while between 10 and 48 hours the reading remains constant. The variation in the final equilibrium attained in the case of these two different samples of blood represents a difference of 1 per cent in hemoglobin concentration. This change in the readings with time may represent the transformation of at least a part of the oxyhemoglobin into some other more stable form. This shift with time makes it necessary that all readings be taken only after the attainment of the final constant transmission value. It has therefore been customary to make readings between 18 and 24 hours after the preparation of the solutions.

To determine the accuracy of the method a number of calibration curves were made for different blood samples. A series of solutions were prepared by dilution of the original 1 : 2,000 solution. These solutions were read by the pyrometer. A calibration curve was then constructed from these values. Care was taken to control the factors of salt content, plasma error, the time factor and errors of the optical system. Two typical calibration curves are illustrated in Fig. 5. Other hemoglobin solutions prepared by appropriate dilution of the original 1 : 2,000 solution were read in the optical pyrometer and their concentration determined by reference to the calibration curve for the same blood sample. A number of such determinations have amply demonstrated that the maximum error is 1 per cent. (Table I.)

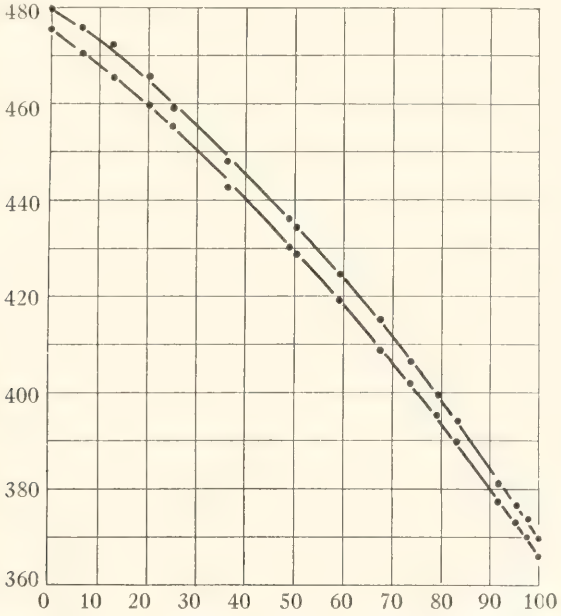

Fig. 5. Two typical calibration curves. Ordinates represent readings of milliammeter; abscissæ, per cent concentration of hemoglobin, 100 per cent being equivalent to a 1 to 2,000 dilution of whole blood (ox).

TABLE I

Data on the Accuracy of the Method

Experiment No.	Percentage of Hemoglobin by Dilution	Percentage of Hemoglobin by Pyrometer	Percentage Error
1	2.5	2.0	−0.5
2	5.0	4.6	−0.4
3	10.0	10.8	+0.8
4	20.0	19.0	−1.0
5	25.0	25.0	0
6	37.5	38.0	+0.5
7	40.0	40.9	+0.9
8	50.0	51.0	+1.0
9	75.0	74.3	−0.7
10	80.0	80.5	+0.5

SUMMARY

The optical pyrometer has been used to determine hemoglobin concentration in dilute solutions with a maximum error of 1 per cent. Accurate determinations can be made with ease and rapidity. The outstanding features of the apparatus are simplicity of construction and a high degree of sensitivity.

Acknowledgment: We wish to thank Dr. D. L. Drabkin for the suggestion which led to the investigation of the shift with time in dilute hemoglobin solutions.

BIBLIOGRAPHY

AMBERSON, W. R., 1922. *Jour. Gen. Physiol.*, **4**: 517.
FOWWEATHER, F. S., 1926. *Biochem. Jour.*, **20**: 93.
PARPART, A. K., 1931. *Biol. Bull.*, **61**: 500.
SCHWENTKER, F. F., 1929. *Jour. Lab. and Clin. Med.*, **15**: 247.
VAN SLYKE, D. D., AND A. HILLER, 1928. *Jour. Biol. Chem.*, **78**: 807.

INDEX

ACTIVATION, total, as related to cleavage in artificially activated Urechis eggs, 45.

ADOLPH, EDWARD F. Body size as a factor in the metamorphosis of tadpoles, 376.

——, ——. The size of the body and the size of the environment in the growth of tadpoles, 350.

Adrenaline, action in elasmobranch fishes, 93.

ALEXANDER, GORDON. The significance of hydrogen ion concentration in the biology of Euglena gracilis Klebs, 165.

AMBERSON, W. R. *See* Parpart, Amberson and Stewart, 518.

Amphiuma tridactyla, oxygen and carbon dioxide transport by blood, 211.

Arbacia, surface tension of eggs, 273.

Asterias eggs, oxygen consumption rate before and after fertilization, 468.

BAUMBERGER, J. P. and L. MICHAELIS. The blood pigments of Urechis caupo, 417.

Blood, of Amphiuma, oxygen and carbon dioxide transport, 211.

——, of marine fishes, respiratory function, 427.

——, of sea lion, respiratory function, 422.

——, respiratory function, in Urechis caupo, 185.

Blood flukes, eggs of, effect of environmental factors on development and hatching, 120.

Blood pigments, of Urechis caupo, 417.

BLUM, H. F. and G. C. MCBRIDE. Studies of photodynamic action, III, 316.

BURKENROAD, M. D. A new pentamerous hydromedusa from the Tortugas, 115.

CARBON dioxide transport by blood of Amphiuma, 211.

Carassius auratus, melanophores in experimental wounds, 73.

CAROTHERS, E. ELEANOR. The maturation divisions and segregation of heteromorphic homologous chromosomes in Acrididae (Orthoptera), 324.

Cellulose, digestion of, by termites, 85.

Cercaria parvicaudata, n. sp., 254.

Cercaria sensifera, n. sp., 259.

Chromosomes of domestic turkey, 157.

Citellus tridecemlineatus pallidus, Allen, laboratory reproduction studies, 101.

Cleavage, as related to total activation in artificially cultivated eggs of Urechis, 45.

COE, WESLEY R. Spermatogenesis in California oyster, 309.

Cytoplasmic contraction in Difflugia, 223.

DEVELOPMENTAL axis, determination of, in Fucus eggs, 294.

DICKMAN, ALBERT. Studies on the intestinal flora of termites with reference to their ability to digest cellulose, 85.

Difflugia, movement and response, 223.

Digestion, of cellulose, by termites, and their intestinal flora, 85.

Dilution of sea water, effect on activity and longevity of marine cercariae, 242.

Diploidism, genetic evidence for, of biparental males in Habrobracon, 139.

——, in male parts in gynandromorphs of Habrobracon, 478.

Drosophila, mutation rate, as affected by continuous and interrupted irradiation, 133.

ELASMOBRANCH fishes, innervation of stomach and rectum and action of adrenaline, 93.

Environment, effect on development and hatching of blood fluke eggs, 120.

——, size of, as affecting body size of tadpoles, 350.

Euglena gracilis Klebs, effect of hydrogen ion concentration on growth, 387.

—— —— ——, significance of hydrogen ion concentration in biology of, 165.

FAULKNER, G. H. Notes on the feeding mechanism and on intestinal respiration in Chaetopterus variopedatus, 472.
Feeding mechanism, Chaetopterus variopedatus, 472.
FLORKIN, M. *See* Redfield and Florkin, 185.
FLORKIN, M., and A. C. REDFIELD. On the respiratory function of the blood of the sea lion, 422.
Fucus, eggs, influence in determination of developmental axis, 294.

GOLDFISH, occurrence of melanophores in experimental wounds, 73.
Ground squirrel, laboratory reproduction studies, 101.
Growth, of Euglena gracilis Klebs, effect of hydrogen ion concentration, 387.
———, of tadpoles, as affected by size of environment, 350.
Gynandromorph, of Habrobracon from post-reduced binucleate egg, 481.
———, of Habrobracon, diploid male parts, 478.

HABROBRACON, diploid male parts in gynandromorphs of, 478.
———, genetic evidence for diploidism in biparental males, 139.
———, gynandromorph from post-reduced binucleate egg, 481.
HALL, F. G. The respiration of puffer fish, 457.
HALL, VICTOR E. The muscular activity and oxygen consumption of Urechis caupo, 400.
HARVEY, E. NEWTON. *See* Taylor and Harvey, 280.
———, ———. The tension at the surface of marine eggs, especially those of the sea urchin, Arbacia, 273.
Hatching, of blood fluke eggs, effect of environmental factors, 120.
Hemoglobin concentration, determination of, in dilute solutions, 518.
Hemolysis, photodynamic, and by non-irradiated eosine, difference in mechanism, 316.
Hydrogen ion concentration, effect on growth of Euglena gracilis Klebs, 387.
———————, significance in biology of Euglena gracilis Klebs, 165.
Hydromedusa, pentamerous, from Tortugas, 115.

INNERVATION of stomach and rectum in elasmobranch fishes, 93.
Intestinal flora of termites, and digestion of cellulose, 85.
Irradiation, continuous and interrupted, effects on mutation rate of Drosophila, 133.

JACOBS, M. H. *See* Williams and Jacobs, 485.
JAHN, THEO. L. Studies on the physiology of the euglenoid flagellates, III, 387.
JOHNSON, GEORGE E. and NELSON J. WADE. Laboratory reproduction studies on the ground squirrel, Citellus tridecemlineatus pallidus, Allen, 101.

LIGHT responses of parasitic water mites, influence of host, 497.
Longevity, certain marine cercariae, as affected by dilution of sea water, 242.
LUTZ, BRENTON R. The innervation of the stomach and rectum and the action of adrenaline in elasmobranch fishes, 93.

MARINE Biological Laboratory, thirty-third report, 1.
MAST, S. O. Movement and response in Difflugia with special reference to the nature of cytoplasmic contraction, 223.
Maturation divisions in Acrididae, 324.
MCBRIDE, G. C. *See* Blum and McBride, 316.
Melanophores, in experimental wounds of goldfish, 73.
Metamorphosis of tadpoles, body size as factor in, 376.
MICHAELIS, L. *See* Baumberger and Michaelis, 417.
Mitogenetic radiation, theory of, 280.
Muscular activity and oxygen consumption of Urechis caupo, 400.
Mutation, rate in Drosophila, effects of continuous and interrupted irradiation, 133.

ONORATO, A. R. and H. W. STUNKARD. The effect of certain environmental factors on the development and hatching of the eggs of blood flukes, 120.

Osmotic hemolysis, question as to whether it is an all-or-none phenomenon, 500.
Ostrea lurida, spermatogenesis in, 309.
Oxygen consumption and muscular activity, Urechis caupo, 400.
—— ——, rate of, by Asterias eggs before and after fertilization, 468.
Oxygen transport, by blood of Amphiuma, 211.
Oyster, California, spermatogenesis, 309.

PARPART, A. K. Is osmotic hemolysis an all-or-none phenomenon? 500.
——, ——, W. R. AMBERSON and D. R. STEWART. The determination of hemoglobin concentration in dilute solutions, 518.
PATTERSON, J. T. Continuous versus interrupted irradiation and the rate of mutation in Drosophila, 133.
Photodynamic action, difference in mechanism between photodynamic hemolysis and hemolysis by irradiated eosine, 316.
Puffer fish, respiration of, 457.

RECTUM, innervation of, elasmobranch fishes, 93.
REDFIELD, A. C. *See* Redfield and Florkin, 422.
——, —— —— —— ——. The respiratory function of the blood of Urechis caupo, 185.
Reproduction studies, in the laboratory, on the ground squirrel, 101.
Respiration, blood pigments of Urechis caupo, 417.
——, function of blood of marine fishes, 427.
——, function of blood of sea lion, 422.
——, function of Urechis caupo, 185.
——, intestinal, in Chaetopterus variopedatus, 472.
——, muscular activity and oxygen consumption of Urechis caupo, 400.
——, oxygen and carbon dioxide transport by blood of Amphiuma tridactyla, 211.
——, oxygen consumption rate of Asterias eggs before and after fertilization, 468.
Response, in Difflugia, 223.
ROOT, R. W. The respiratory function of the blood of marine fishes, 427.

SCOTT, W. J. Oxygen and carbon dioxide transport by the blood of the urodele, Amphiuma tridactyla, 211.
Sea lion, respiratory function of blood, 422.
Sea water, dilution of, effect on activity and longevity of marine cercariae, 242.
Segregation of heteromorphic homologous chromosomes in Acrididae, 324.
Size, of body and of environment in growth of tadpoles, 350.
Size, of body in metamorphosis of tadpoles, 376.
SMITH, GEORGE MILTON. The occurrence of melanophores in certain experimental wounds of the goldfish (Carassius auratus), 73.
Sodium chloride, physiological differences between different preparations, 485.
Spermatogenesis, of California oyster, 309.
STANCATI, M. F. *See* Whiting and Stancati, 478.
STEWART, D. R. *See* Parpart, Amberson and Stewart, 518.
Stomach, innervation of, elasmobranch fishes, 93.
STUNKARD, H. W. *See* Onorato and Stunkard, 120.
——, ——. The effect of dilution of sea water on the activity and longevity of certain marine cercariae, 242.
Surface tension marine eggs, especially Arbacia, 273.

TADPOLES, body size and environment size, 350.
——, body size in metamorphosis of, 376.
TANG, PEI-SUNG. The rate of oxygen consumption of Asterias eggs before and after fertilization, 468.
TAYLOR, G. WELLFORD and E. NEWTON HARVEY. The theory of mitogenetic radiation, 280.
Termites, their intestinal flora with reference to digestion of cellulose, 85.
Thirty-third report of the Marine Biological Laboratory, 1.
TORVIK, M. M. Genetic evidence for diploidism of biparental males in Habrobracon, 139.
Turkey, domestic, chromosomes of, 157.
TYLER, ALBERT. The relation between cleavage and total activation in arti-

ficially cultivated eggs of Urechis, 45.

URECHIS, relation between cleavage and total activation in artificially cultivated eggs, 45.

Urechis caupo, blood pigments of, 417.

———, muscular activity and oxygen consumption, 400.

———, respiratory function of blood, 185.

WADE, NELSON J. *See* Johnson and Wade, 101.

Water mites, parasitic, influence of host on light responses, 497.

WELSH, JOHN H. Specific influence of the host on the light responses of parasitic water mites, 497.

WERNER, ORILLA STOTLER. The chromosomes of the domestic turkey, 157.

WHITAKER, D. M. Some observations on the eggs of Fucus and upon their mutual influence in the determination of the developmental axis, 294.

WHITING, P. W. Diploid male parts in gynandromorphs of Habrobracon, 478.

WHITING, P. W. and M. F. STANCATI. A gynandromorph of Habrobracon from a post-reduced binucleate egg, 481.

WILLIAMS, MARY MORRISON and M. H. JACOBS. On certain physiological differences between different preparations of so-called "chemically pure" sodium chloride, 485.

Volume LXI Number 1

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CONTENTS

Page

THIRTY-THIRD REPORT OF THE MARINE BIOLOGICAL LABORATORY 1

TYLER, ALBERT
The Relation between Cleavage and Total Activation in Artificially Activated Eggs of Urechis 45

SMITH, GEORGE MILTON
The Occurrence of Melanophores in certain Experimental Wounds of the Goldfish (Carassius auratus) 73

DICKMAN, ALBERT
Studies on the Intestinal Flora of Termites with reference to their Ability to Digest Cellulose 85

LUTZ, BRENTON R.
The Innervation of the Stomach and Rectum and the Action of Adrenaline in Elasmobranch Fishes 93

JOHNSON, GEORGE E., AND NELSON J. WADE
Laboratory Reproduction Studies on the Ground Squirrel, Citellus tridecemlineatus pallidus, Allen 101

BURKENROAD, M.D.
A New Pentamerous Hydromedusa from the Tortugas 115

ONORATO, A. R., AND H. W. STUNKARD
The Effect of certain Environmental Factors on the Development and Hatching of the Eggs of Blood Flukes 120

Volume LXI Number 2

THE
BIOLOGICAL BULLETIN

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Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

CONTENTS

Page

PATTERSON, J. T.
Continuous versus Interrupted Irradiation and the Rate of Mutation in Drosophila 133

TORVIK, M. M.
Genetic Evidence for Diploidism of Biparental Males in Habrobracon 139

WERNER, ORILLA STOTLER
The Chromosomes of the Domestic Turkey 157

ALEXANDER, GORDON
The Significance of Hydrogen Ion Concentration in the Biology of Euglena gracilis Klebs 165

REDFIELD, A. C., AND M. FLORKIN
The Respiratory Function of the Blood of Urechis caupo 185

SCOTT, W. J.
Oxygen and Carbon Dioxide Transport by the Blood of the Urodele, Amphiuma tridactyla 211

MAST, S. O.
Movement and Response in Difflugia with special reference to the Nature of Cytoplasmic Contraction 223

STUNKARD, H. W.
The Effect of Dilution of Sea Water on the Activity and Longevity of Certain Marine Cercariae 242

Volume LXI
Number 3

THE
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Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

CONTENTS

Page

HARVEY, E. NEWTON
The Tension at the Surface of Marine Eggs, especially those of the Sea Urchin, Arbacia 273

TAYLOR, G. WELLFORD, AND E. NEWTON HARVEY
The Theory of Mitogenetic Radiation 280

WHITAKER, D. M.
Some Observations on the Eggs of Fucus and upon their Mutual Influence in the Determination of the Developmental Axis 294

COE, WESLEY R.
Spermatogenesis in the California Oyster (Ostrea lurida) 309

BLUM, H. F., AND G. C. MCBRIDE
Studies of Photodynamic Action, III. The difference in mechanism between photodynamic hemolysis and hemolysis by non-irradiated eosine 316

CAROTHERS, E. ELEANOR
The Maturation Divisions and Segregation of Heteromorphic Homologous Chromosomes in Acrididae (Orthoptera) 324

ADOLPH, EDWARD F.
The Size of the Body and the Size of the Environment in the Growth of Tadpoles 350

ADOLPH, EDWARD F.
Body Size as a Factor in the Metamorphosis of Tadpoles 376

JAHN, THEO. L.
Studies on the Physiology of the Euglenoid Flagellates, III. The effect of hydrogen ion concentration on the growth of Euglena gracilis Klebs 387

HALL, VICTOR E.
The Muscular Activity and Oxygen Consumption of Urechis caupo .. 400

BAUMBERGER, J. P., AND L. MICHAELIS
The Blood Pigments of Urechis caupo 417

FLORKIN, MARCEL, AND ALFRED C. REDFIELD
On the Respiratory Function of the Blood of the Sea Lion 422

ROOT, R. W.
The Respiratory Function of the Blood of Marine Fishes 427

HALL, F. G.
The Respiration of Puffer Fish 457

TANG, PEI-SUNG
The Rate of Oxygen Consumption of Asterias Eggs Before and After Fertilization 468

FAULKNER, G. H.
Notes on the Feeding Mechanism and on Intestinal Respiration in Chaetopterus variopedatus 472

WHITING, P. W.
Diploid Male Parts in Gynandromorphs of Habrobracon 478

WHITING, P. W., AND M. F. STANCATI
A Gynandromorph of Habrobracon from a Post-Reduced Binucleate Egg 481

WILLIAMS, MARY MORRISON, AND M. H. JACOBS
On Certain Physiological Differences between Different Preparations of So-Called " Chemically Pure " Sodium Chloride 485

WELSH, JOHN H.
Specific Influence of the Host on the Light Responses of Parasitic Water Mites 497

PARPART, ARTHUR K.
Is Osmotic Hemolysis an All-or-none Phenomenon? 500

PARPART, A. K., W. R. AMBERSON AND D. R. STEWART
The Determination of Hemoglobin Concentration in Dilute Solutions 518

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